The use of statins in acute myeloid leukemia

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1

General introduction

INTRODUCTION

Despite intensive treatment, long term survival rates of patients suffering from acute myeloid leukemia (AML) are still low. A major cause is resistance to chemotherapy, that can be mediated by overexpression of drug-effluxing transporters, but a role for dysregulated cholesterol metabolism has also been suggested. Blocking cholesterol synthesis using statins, cholesterol-lowering drugs widely used for the prevention of cardiovascular disease, has been proposed as a promising approach to improve current antileukemic treatment. Work described in this thesis explores the use of statins in AML, with a focus on cotreatment with other agents and on the mechanism(s) of action.

Normal and leukemic hematopoiesis

Normal hematopoiesis comprises the formation of the different blood cell lineages during the life-span of an organism. This is a hierarchically organized process in which the most undifferentiated hematopoietic stem cell (HSC) gives rise to (multipotent) progenitor cells (Figure 1). These progenitor cells differentiate to mature blood cells of different lineages. In addition, HSCs can give rise to new HSCs, a process called

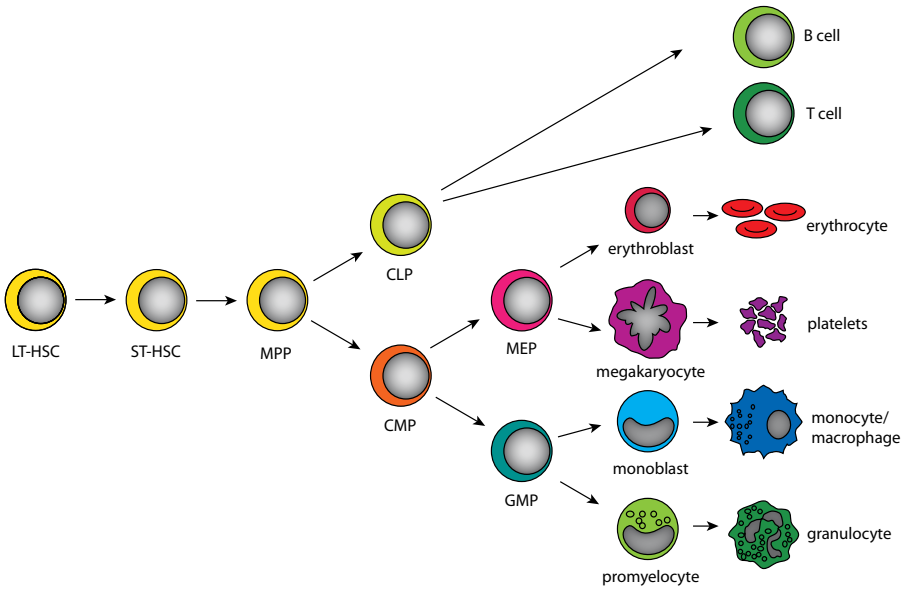

Figure 1. Hierarchical structure of hematopoiesis. Schematic representation of hematopoiesis displaying the development of mature blood cell lineages from hematopoietic stem cells. LT-HSC: long-term hematopoietic stem cell; ST-HSC: short-term HSC; MPP: multipotent progenitor; CMP: common myeloid progenitor; CLP: common lymphoid progenitor; GMP: granulocyte/macrophage progenitor; MEP: megakaryocyte/erythrocyte progenitor.

self-renewal[1,2], which underlies the capacity to repopulate the bone marrow of irradiated recipients[3]. The processes of self-renewal, proliferation, and differentiation need to be tightly regulated, as dysregulation of hematopoiesis might result in severe defects in the generation of one or more of the cell lineages.

In contrast to normal hematopoiesis, leukemic hematopoiesis is characterized by a block in differentiation capacity in combination with defects in self-renewal and cell survival, which ultimately results in the accumulation of myeloid blasts in the bone marrow and suppression of normal hematopoiesis[4-7]. Researchers have proposed that within AML a hierarchical structure similar to that of normal hematopoiesis is present and that the leukemic stem cell (LSC), the leukemic counterpart of the HSC, resides on top of this hierarchy[2,8,9]. However, recently cells with leukemia-initiating capacity were also found among cells that display expression markers that are associated with normal committed progenitors, indicating that leukemia-initiating cells are not restricted to the phenotype associated with normal HSCs[10].

Stem cells

Normal and leukemic stem cells are characterized by the expression of the membrane antigen CD34 and the lack of CD38 ($CD34^+CD38^-$)[3,11-15], although it has recently been shown that also the $CD34^+CD38^+$ and even the $CD34^-$ cell fraction contain leukemia-initiating cell capacity[16,17]. There is increasing evidence that LSCs play an important role in the leukemogenic process[18]. In murine models, AML arises in HSCs by leukemic transformation, rather than in committed progenitors[15,18], with the exception of acute promyelocytic leukemia, where transformation takes place in more committed cells[19]. However, recent reports show that LSCs are phenotypically more similar to normal progenitor populations than to stem cells[20], and that, in contrast to a hierarchical structure, leukemias reveal a non-linear branching clonal architecture with subclones having distinctive genetic signatures[21]. During leukemic transformation, two separate events can be distinguished: leukemic initiation and maintenance. It is thought that both events are interconnected and dependent on multiple events that ultimately lead to enhanced self-renewal and differentiation defects of the LSC. Not only intrinsic defects like gene mutations in the *RAS*, *C-KIT,* or *BCR/ABL* gene are part of the transformation[22-24], but also extrinsic factors, like increased production of cytokines and growth factors[4,25-27]. In addition, localization in and interaction with the bone marrow microenvironment play a role in the development and maintenance of AML[28]. Although chemotherapy is able to eradicate the dividing AML blast cells, it may not be effective for elimination of the LSC population, as this population is relatively quiescent and may have acquired resistance[29,30].

Microenvironment

Normal and leukemic stem cells reside in the bone marrow surrounded by their microenvironment. This so-called niche consists of two separate regions; the endosteal niche, that is composed of osteoblasts, osteoclasts and stromal cells, and the vascular niche, a region closer to the vasculature that comprises sinusoid endothelial cells. The cells of the niche allow interaction with HSCs and provide important hematopoietic cytokines, both of which are essential for survival and maintenance of HSCs[31].

In the bone marrow microenvironment oxygen levels are low (1-6%) in comparison with the levels in the bloodstream (21%)[32]. At these low oxygen levels, cells switch to glycolysis for their energy supply. This results in a decreased generation of ATP, which is thought to slow down cell proliferation and to sustain the quiescent state of HSCs[33]. As HSCs and progenitors migrate towards the more oxygen-rich regions of the bone marrow and the vasculature, proliferation and differentiation of these cells are favored.

Acute myeloid leukemia

AML is the most common acute leukemia in adults, and its incidence increases with age. There are different subtypes of AML; for classification of AML the World Health Organization (WHO) system is currently being used in the clinic. This system categorizes leukemia especially according to cytogenetic abnormalities[34]. These abnormalities provide prognostic information resulting in the categorization of AML patients in different risk groups.

Patients with AML are treated with intensive chemotherapy, which consists frequently of the combination of cytarabine (ARA-C) and an anthracycline (e.g., daunorubicin), aimed at induction of a remission. At older age (>70-75 years) palliative care is offered instead. After having obtained a complete remission, consolidation therapy is applied, which consists of an additional course of chemotherapy, but may include a stem cell transplantation as well, depending on the risk group the patient belongs to[35,36]. The chances of full recovery from the disease for a specific patient depend on a number of prognostic factors including cytogenetics[37] and gene mutations[38,39]. Due to intensive treatment options, 50% of the good-risk patients are cured, which contrasts with only 4% of AML patients belonging to the unfavorable risk group[40]. For patients over 65 years the figures are even lower[41].

Cholesterol

Cholesterol is an essential molecule for cells as a major constituent of all cellular membranes. Besides being required for the formation of new membranes during cell division, cholesterol content influences membrane organization and, thereby, membrane properties. Cholesterol is often clustered in microdomains within

plasma membranes that are commonly referred to as lipid rafts[42]. Lipid rafts play an important role in signal transduction. Furthermore, cholesterol is a precursor for steroid hormones, bile acids, and some vitamins.

Cellular cholesterol homeostasis is maintained by tightly controlled mechanisms of *de novo* cholesterol synthesis, cellular influx, and cellular efflux (Figure 2). Cholesterol is synthesized from acetyl coenzyme A (acetyl-CoA) moieties in a number of steps via the mevalonate pathway, and involves the action of the rate-controlling enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoAR). This mevalonate pathway is described below in more detail. Influx of cholesterol in the form of LDL (low-density lipoprotein) is mediated by LDL receptor (LDLR). LDLR expression is regulated by sterol response element-binding protein 2 (SREBP2), and is induced when cellular cholesterol levels are low[43,44]. In this way cellular cholesterol levels exhibit a negative feedback on cholesterol influx[45]. In addition, scavenger receptor-BI (SR-BI) takes up cholesterol esters from high-density lipoprotein (HDL)[46]. Cholesterol efflux is induced by liver X receptor (LXR), of which two isoforms have been identified (LXRα and LXRβ). LXR is activated by oxysterols, oxygenated derivatives of cholesterol, and, upon heterodimerization with retinoid X receptor (RXR), induces the transcription of *ABCA1* and *ABCG1,* and other target genes involved in cholesterol transport[47-49].

ABCA1 and ABCG1 belong to the superfamily of adenosine triphosphate (ATP)-binding cassette (ABC) transporters, and are known to mediate cellular cholesterol efflux towards acceptors such as ApoA-I and high-density lipoproteins (HDL)[50-52]. The ABC superfamily of transporters comprises 49 functionally distinct transmembrane proteins that transport compounds across plasma and intracellular membranes. The transporters can be divided in 7 subfamilies (ABCA through ABCG). Although some of these transporters efflux specific compounds, many of them are able to extrude a variety of structurally unrelated compounds. Hydrolysis of ATP provides the transporters with energy to actively export natural and chemical (drugs) compounds across cell membranes against concentration gradients. A number of ABC transporters is highly expressed at the mRNA level in normal HSCs, including the ones involved in cholesterol transport[53-57].

Cholesterol in AML

Over 40 years ago a relationship between aberrant cholesterol metabolism and cancer was identified[58]. In 1978, Goldstein and Brown were the first to report altered cholesterol metabolism in AML[59]. In AML cells mRNA levels of HMG-CoAR and LDLR are increased ,and activity of these proteins is higher compared with normal bone marrow cells[60,61]. Additional studies revealed that AML cells often do not display efficient feedback repression of cholesterol synthesis and influx when exposed to high-cholesterol media

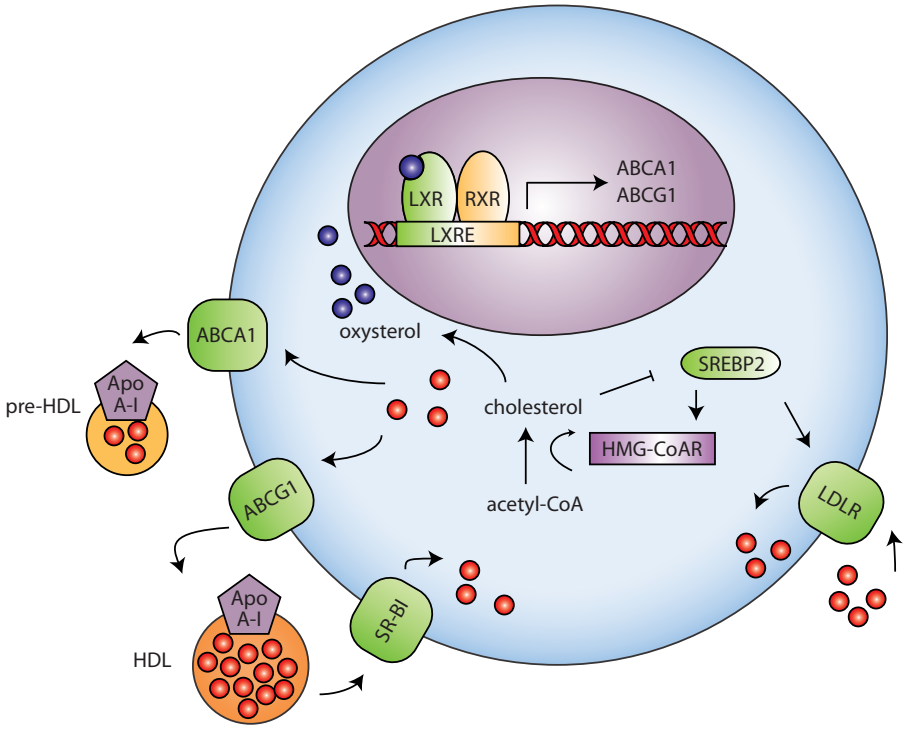

Figure 2. Schematic representation of cellular cholesterol homeostasis. LDLR: low-density lipoprotein (LDL) receptor; SREBP2: sterol response element-binding protein-2; acetyl-CoA: acetyl coenzyme A; HMG-CoAR: 3-hydroxy-3-methyl-glutaryl-CoA reductase; LXR: liver X receptor; RXR: retinoid X receptor; LXRE: LXR response element; ABCA1 and ABCG1: ATP-binding cassette transporter A1 and G1; ApoA-I: apolipoprotein A-I; HDL: high-density lipoprotein; SR-BI: scavenger receptor BI.

in vitro. This feature appeared to be associated with increased survival[62,63]. In addition, AML cells from a subgroup of patients showed *in vitro* an acute increase in cellular cholesterol content in response to chemotherapy (DNR, ARA-C) exposure[63]. This cholesterol increment could be (partly) prevented by cholesterol synthesis inhibitors, and this, surprisingly, resulted in an improved chemosensitivity[62,64]. These findings suggest a role for cholesterol inhibitors to improve standard antileukemic treatment.

Mevalonate pathway

Cholesterol is synthesized from acetyl-COA moieties by the mevalonate pathway (Figure 3). The initial step in this complex synthetic pathway is the formation of HMG-COA from acetyl-COA and acetoacetyl-COA, which is catalyzed by HMG-COA synthase. Subsequently, HMG-COA is converted to mevalonate by

HMG-CoAR, representing the rate-controlling step of the mevalonate pathway. Mevalonate is subsequently converted in several steps to farnesyl pyrophosphate (FPP). FPP then can be converted to squalene and subsequently, in multiple steps, to cholesterol. Oxygenated forms of cholesterol, oxysterols, bind to the nuclear transcription factor LXR, which activates the transcription of ABCA1 and ABCG1, amongst others.

Besides a cholesterol synthesis branch, the mevalonate pathway also comprises an isoprenylation branch. The earlier mentioned isoprenoid FPP can be converted to another isoprenoid, geranylgeranyl pyrophosphate (GGPP). Both isoprenoids are utilized for the isoprenylation of small guanosine triphosphate hydrolases (GTPases), such as Ras and Rho, which are involved in control of proliferation, signal transduction, and apoptosis[65-68]. Covalent attachment of a farnesyl group (farnesylation) or a geranylgeranyl group (geranylgeranylation) to small GTPases is catalyzed by farnesyltransferase (FTase) or geranylgeranyltransferase I (GGTase), respectively. Rho can only be geranylgeranylated[69], whereas H-Ras is exclusively farnesylated, and K-Ras and N-Ras can be both farnesylated and geranylgeranylated[70]. Isoprenylation is a posttranslational modification that is necessary for plasma membrane binding and

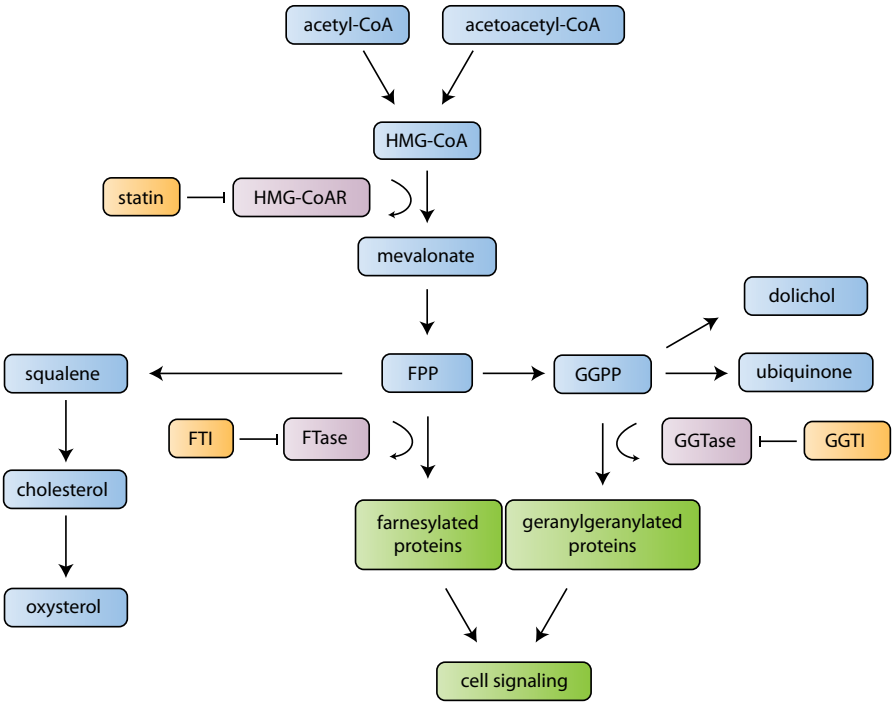

Figure 3. Schematic representation of the mevalonate pathway. HMG-CoAR: 3-hydroxy-3-methyl-glutaryl-CoA reductase; FPP: farnesyl pyrophosphate; FTase: farnesyltransferase; FTI: FTase inhibitor; GGPP: geranylgeranyl pyrophosphate; GGTase: geranylgeranyltransferase; GGTI: GGTase inhibitor.

the subsequent activity and participation in signal transduction of small GTPases. Other products of the mevalonate pathway include dolichol, required for glycoprotein synthesis, and ubiquinone, that is involved in oxidative respiration[65].

Signal transduction

Constitutive activation of Ras is implicated in malignant growth of hematopoietic cells and activating mutations, predominantly in *N-RAS* and less often in *K-RAS*, are found in 15-30% of AML cases[67,71]. In addition, deregulation of Rho-GTPases is associated with hematological disease, including AML[72]. Ras- and Rho-GTPases are the most well-described subgroups of the Ras family of small GTPases. The Ras subgroup contains Ras, Rap1 and Ral[73], whereas the Rho-GTPases are Rho, Rac, and CDC42[74].

GTPases function as molecular switches within a cell, cycling between an active, GTP-bound state and an inactive, GDP-bound state. Upon stimulation of cells, e.g., by cytokines or growth factors, the GTPase is activated by exchanging GDP for GTP, which leads to the activation of downstream signaling pathways. Different small GTPases have separate functions in cells, which may even differ within cell types. Ras, for example, mediates proliferation, differentiation and apoptosis by activating the Raf/MEK/ERK pathway and/or the PI3K/Akt pathway[75,76]. Rho is mostly known for its role in cytoskeleton function[77], although it is also involved in cell adhesion and proliferation, cell migration, apoptosis and survival, cell cycle progression, and genomic stability[72,78,79].

Statins

Statins inhibit the mevalonate pathway by competitively inhibiting HMG-CoAR. Several HMG-CoAR inhibitors have been developed for clinical use, four of which are naturally occurring and/or derived from fungal fermentation (simvastatin, lovastatin, pravastatin, and mevastatin), whereas the remaining five are synthetic (atorvastatin, cerivastatin, fluvastatin, pitavastatin, and rosuvastatin). Statins are clinically used for the treatment of hypercholesterolemia (10-80 mg/day), as statins reduce plasma cholesterol levels, thereby preventing the development of atherosclerotic plaques and consequent cardiovascular disease[80]. However, because additional 'pleiotropic' effects have been attributed to statins due to blockage of the mevalonate pathway, statins are also explored for alternative indications, including treatment strategies for certain malignancies[81]. Several (pre)clinical trials have been done to investigate the potential beneficial effects of statins in AML[82-85]. These trials showed that statins can be given to cancer patients in relatively high dosages, i.e., 25 mg/kg/day for lovastatin and 15 mg/kg/day for simvastatin[82,85].

Scope of the thesis

Based on the finding that cholesterol metabolism is dysregulated in AML and the possible additional effects of statins for treatment, this thesis aims to explore the actions of statins in AML. We will focus on cotreatment with other agents and on the mechanism(s) involved, since targeting the mevalonate pathway by statins also interferes with signaling events and thus may have potential relevance for the treatment of AML. *Chapter 2* reviews recent developments in the role of cholesterol and the mevalonate pathway and in intervention with this pathway in AML. As statins are reported to sensitize AML cells to chemotherapy, we investigated in *chapter 3* whether lovastatin could potentiate chemotherapy-induced cytotoxicity in the primitive $CD34^+$ fraction of AML cells. This $CD34^+$ fraction is less susceptible to chemotherapy due to its relative quiescent state, and has a higher cholesterol turnover than more mature cells. We assessed viability and colony forming cell frequency upon treatment of primary AML $CD34^+$ cells with either lovastatin, chemotherapy, or the combination. Likewise, we studied in *chapter 4* whether the effects of another statin, simvastatin, could be promoted by cotreating $CD34^+$ AML cells with tipifarnib, a farnesyltransferase inhibitor. Both compounds act on the mevalonate pathway, and are already being tested in clinical settings. Alternative prenylation by geranylgeranyltransferase may bypass the inhibitory effects of tipifarnib. In addition, statins are, in theory, capable of blocking both geranylgeranylation and farnesylation. Therefore, the combined use of tipifarnib with simvastatin may have more pronounced antileukemic effects. We assessed viability of AML cell lines and primary $CD34^+$ cells upon treatment with simvastatin combined with tipifarnib. In *chapters 3* and *4*, we noted that some AML patient samples responded well to statin treatment, whereas other samples were not responsive at all. In *chapter 5*, we tried to elucidate the mechanism behind this heterogeneity in responsiveness. We used AML cell lines and primary AML cells with different sensitivities to simvastatin and investigated the role of the cholesterol synthesis route and the isoprenylation synthesis route of the mevalonate pathway in these heterogeneous effects. To translate our *in vitro* findings to an *in vivo* setting, we studied samples of AML patients treated with simvastatin, which is described in c*hapter 6*. In addition, mice were studied in order to have additional access to liver tissue, which is the primary target of statins, as to compare our findings in liver cells with those in bone marrow. We assessed the *in vivo* effects of simvastatin treatment on the different routes of the mevalonate pathway by gene expression analysis and protein Western blots. Finally, *chapter 7* summarizes the main findings of the thesis and discusses implications for future research and clinical application.

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Therapeutic potential of targeting the mevalonate pathway in acute myeloid leukemia

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ABSTRACT

The mevalonate pathway is of crucial importance for the maintenance of cellular cholesterol homeostasis. It takes care of adequate cholesterol production to balance variations in cholesterol uptake and efflux. This pathway also generates isoprenoid moieties, i.e., farnesyl and geranylgeranyl pyrophosphates that are indispensable for the function of small GTPases, such as Ras and Rho. There is a growing interest in the role of cholesterol and intermediates of the mevalonate pathway in acute myeloid leukemia (AML). Recently, important roles for cholesterol transporting ABC transporters and for high-density lipoproteins in hematopoietic stem cell proliferation have been demonstrated. In addition, therapies aimed to block cholesterol synthesis result in cholesterol-independent effects that may be beneficial in the treatment of AML patients. However, clinical studies on statin use in AML have shown thus far unsatisfactory results. This review describes the recent advances in our understanding of cholesterol metabolism and the mevalonate pathway in hematopoietic disorders, with a focus on AML. Additionally, it evaluates the consequences of (pre)clinical intervention in these processes. Also, alternative and thus far underexposed implications of statin treatment are discussed, such as the effects on the bone marrow microenvironment and on the occurrence of graft-versus-host disease.

Introduction

Acute myeloid leukemia (AML) is a hematopoietic disorder characterized by the accumulation of immature myeloid blasts in the bone marrow and suppression of normal hematopoiesis. The accumulation of blasts is caused by a block in differentiation capacity in conjunction with defects in self-renewal and cell survival[1-4]. Within AML there are several subtypes that can be distinguished by chromosomal and molecular markers[5]. These characteristics serve as prognostic factors, allowing to categorize the patients into favorable, intermediate, or unfavorable risk groups[6,7]. Despite intensive treatment, only 30-40% of AML patients between 18 and 65 years old are cured and for patients over 65 years this figure is only 5-10%[8]. A major problem in the treatment of AML is the intrinsic or acquired resistance to chemotherapy. One of the causes of this multidrug resistance is a dysregulation of cellular cholesterol homeostasis[9].

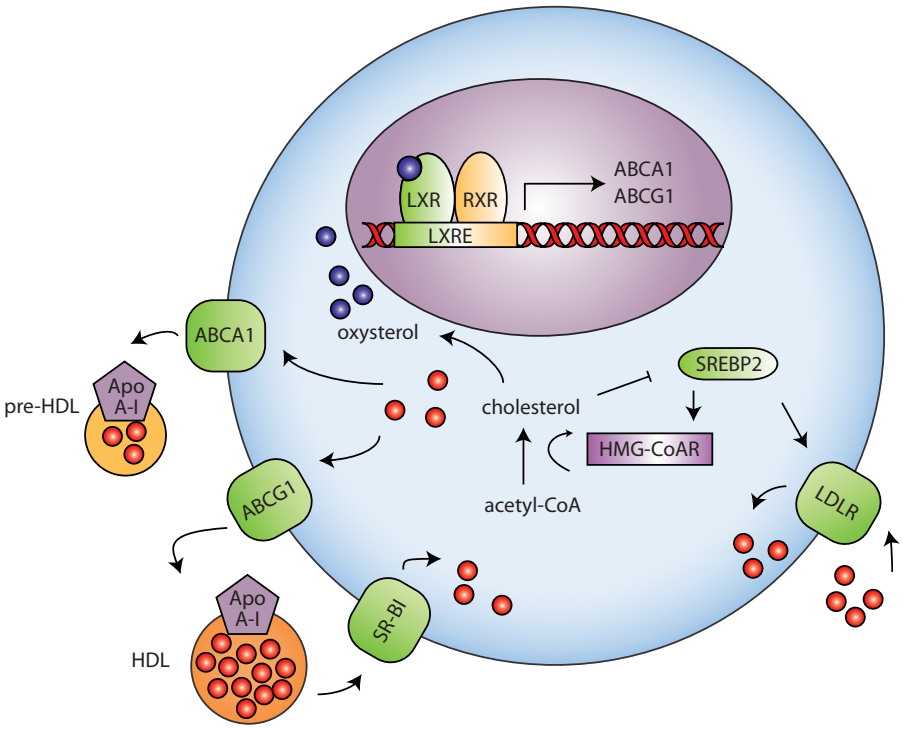

Figure 1. Schematic representation of cellular cholesterol homeostasis. LDLR: low-density lipoprotein (LDL) receptor; SREBP2: sterol response element-binding protein-2; acetyl-CoA: acetyl coenzyme A; HMG-CoAR: 3-hydroxy-3-methyl-glutaryl-CoA reductase; LXR: liver X receptor; RXR: retinoid X receptor; LXRE: LXR response element; ABCA1 and ABCG1: ATP-binding cassette transporter A1 and G1; ApoA-I: apolipoprotein A-I; HDL: high-density lipoprotein; SR-BI: scavenger receptor BI.

More than 40 years ago, a relationship between cholesterol metabolism and cancer was first suggested[10], which was in 1978 shown for AML[11]. Both synthesis and influx of cholesterol are increased in patients' AML cells. This can be attributed to an increased mRNA expression and protein activity of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoAR), the rate-controlling enzyme of the mevalonate pathway, and low-density lipoprotein receptor (LDLR), responsible for cholesterol influx (Figure 1)[12]. Moreover, leukemic cells do not exhibit adequate feedback repression of these genes upon exposure to excess cholesterol[13]. AML cells increase their cholesterol contents after *in vitro* exposure to chemotherapeutic drugs, which might render them less susceptible to the effects of these agents. In addition, cholesterol-effluxing ABC transporters play a role in hematopoietic cell proliferation[14]. Considering these findings, interfering with cholesterol homeostasis may offer an opportunity to improve antileukemic treatment[12].

The most widely used cholesterol-lowering agents are statins, cholesterol synthesis inhibitors developed for the prevention of cardiovascular diseases. Based on the above described findings, statins have also been proposed for treatment of AML. Statins underwent clinical testing with dosages up to 50-fold higher than used to treat hypercholesterolemia. However, it has become increasingly evident that not the effects on cholesterol synthesis, but rather the 'pleiotropic' effects, i.e., interference with isoprenoid synthesis, are most relevant for statin-mediated preclinical antitumor effects[15-18]. This review aims to picture current knowledge of the role of the mevalonate pathway in AML and addresses recent developments and (pre)clinical experience with the intervention with this pathway by statins.

The role of extracellular and intracellular cholesterol in AML

Cholesterol is an important component of cell membranes and essential for growth and proliferation of normal as well as cancer cells[19,20]. It is required for the formation of cell membranes of dividing cells, as well as the formation of lipid rafts that are crucial for adequate signal transduction. Elevated plasma cholesterol levels modulate features of bone m arrow cells. For example, in mice a high cholesterol diet resulting in elevated plasma LDL levels is associated with increased bone marrow cell proliferation[21]. This is likely due to an increase of cholesterol content in lipid rafts. Human breast and prostate cancer cell lines display higher levels of cholesterol-rich lipid rafts than their normal counterparts[22]. In addition, in human prostate cancer xenografts in SCID mice elevated serum cholesterol levels increase the cholesterol content in lipid rafts of tumor cells, thereby affecting tumor cell signaling, promoting tumor growth and reducing apoptosis[23].

Seemingly conflicting with these observations is the finding that AML patients generally have low plasma cholesterol levels[24]. A retrospective study showed that

low serum LDL cholesterol levels (≤ 70 mg/dL) are associated with an increased risk to develop hematologic malignancy[25]. However, these low LDL cholesterol levels are likely the effect instead of the cause of malignant cell growth. A recent study in acute lymphoid leukemia (ALL) patients revealed that primary lymphocytes contain high levels of cholesterol despite low serum cholesterol levels[26]. Also AML cells display increased LDL receptor-mediated uptake of cholesterol in conjunction with lower plasma cholesterol levels[27]. This suggests a higher cholesterol consumption for tumor cell proliferation, which is supported by the finding that after chemotherapy, when the leukemic cells are absent, plasma cholesterol levels normalized[27].

Whereas high LDL levels are associated with increased cell proliferation[21], high levels of high-density lipoprotein (HDL), responsible for picking up effluxed cholesterol, decrease proliferation of hematopoietic cells[14]. The excessive proliferation of myeloid cells containing high cholesterol levels in *Abca1*/*Abcg1* double knockout (KO) mice could be reversed by transplanting bone marrow cells of these mice into *Apoa-I* transgenic mice that display high HDL levels[14]. This observation, which showed that high HDL levels were able to suppress proliferation in the absence of ABCA1 and ABCG1, indicates that HDL can promote cholesterol efflux by alternative pathways, like passive cholesterol efflux[28].

Exposure of patient AML cells to cytotoxic drugs, such as cytarabine and daunorubicin, causes an increase in LDLR and HMG-CoAR mRNA levels in many human AML cell samples, resulting in increased cellular cholesterol levels[9,29]. This is thought to protect cells against cytotoxic effects, contributing to leukemic therapy failure in part of the AML patients[30]. It is uncertain how these increases of mRNA levels relate to protein activity, and whether the chemotherapy-mediated cholesterol increments are caused by accelerated influx of LDL or by increased *de novo* synthesis. LDL accumulation did not correlate with cholesterol increments in cultured cytarabine-treated AML cells[9]. This suggests that cholesterol increments do not result from high cholesterol influx and that probably *de novo* synthesis of cholesterol is involved[9]. In addition, in contrast to cytarabine treatment, *in vitro* daunorubicin treatment-mediated induction of HMG-CoAR and LDLR mRNA levels in AML cells did not correlate with cholesterol increments. These findings indicate that drugs differ with respect to their ability to induce cholesterol responses in AML cells, and that posttranscriptional regulation contributes to the adaptive responses[9]. However, it is clear that cholesterol plays a role in the response to chemotherapy. In a clinical trial in AML patients, two distinct response patterns were observed upon treatment with the cholesterol synthesis inhibitor pravastatin[30]. Here, serum cholesterol levels either permanently decreased or an initial decrease was followed by a rebound to pretreatment levels. These rebounds in cholesterol levels were associated with resistance to chemotherapy; patients achieving a

complete remission (CR) upon combined idarubicin and high-dose cytarabine showed no rebound in serum cholesterol levels[30]. However, the mechanism responsible for the rebound in resistant patients remains to be investigated.

Esterified cholesterol (cholesteryl ester) can be stored in adipocytes. Adipocytes are constituents of the bone marrow that increase in number upon aging. They have not been intensively investigated thus far, likely because they are perceived as 'passive space fillers'. However, more recently fascinating advances have been made. Besides bone marrow, also adipose tissue was identified as a reservoir for functional hematopoietic stem and progenitor cells[31]. In addition, the number of adipocytes in bone marrow was inversely correlated with hematopoietic activity of the bone marrow: in adipocyte-poor bone marrow more progenitors in the S/G2/M phase of cell cycle were found[32]. As in AML patients irradiation or chemotherapy is usually followed by fatty infiltration of the bone marrow, this can delay marrow recovery after stem cell transplantation: engraftment in mice is higher and faster if the bone marrow contains few or no adipocytes[32]. The more efficient engraftment in adipocyte-poor bone marrow can be explained by a concomitant induction of osteogenesis, promoting a more supportive environment for hematopoietic reconstitution[33]. Additionally, adipocytes protect leukemia cells against the cytotoxic effects of chemotherapy[34]. This may be caused by the fact that adipocytes accumulate hydrophobic drugs, or, more likely, that in the presence of adipocytes leukemic cells increase the expression and activation of anti-apoptotic proteins such as Bad, Bcl-2 and Pim2[34].

The mevalonate pathway: the cholesterol synthesis route and the isoprenylation route

Maintenance of intracellular cholesterol homeostasis is achieved by controlling cholesterol influx by LDLR, its efflux by ABC transporters ABCA1 and ABCG1, and via regulation of *de novo* cholesterol synthesis**.** The mevalonate pathway is of crucial importance for cellular cholesterol homeostasis, as cholesterol is synthesized via this pathway (Figure 2). In addition, oxidized derivatives of cholesterol (oxysterol, such as 24(*S*),25-epoxycholesterol, 20(*S*)-hypoxycholesterol (20(*S*)-HC), 22(*R*)-HC, 24(*S*)-HC and 27-HC[35]), can activate the nuclear receptor liver X receptor (LXR). This results in the expression of target genes that control cellular cholesterol (e.g., *ABCA1* and *ABCG1*) and fatty acid metabolism (e.g., *SREBP-1C* and fatty acid synthase)[36]. ABCA1 mediates efflux of excess cellular cholesterol to apolipoprotein A-I (ApoA-I) and nascent HDL, whereas ABCG1 mediates efflux of cholesterol to more mature HDL particles (Figure 1). Uptake of cholesterol is mediated by LDLR, the expression and activity of which is regulated in a complex fashion independent of the mevalonate pathway (reviewed by Goldstein and Brown[54]).

Apart from cholesterol and oxysterols, the mevalonate pathway also leads to the production of the isoprenoids farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (Figure 2). FPP is a precursor for ubiquinone and dolichol. Ubiquinone, also called coenzyme Q_{10}, is an anti-oxidant that serves as an electron shuttle in mitochondria, whereas dolichol plays a role in N-glycosylation of proteins. In addition, FPP and GGPP are essential for isoprenylation of proteins, a posttranslational modification that is necessary for biological activity of small guanosine triphosphate hydrolases (GTPases) like Ras and Rho. Isoprenylation is catalyzed by farnesyltransferase (FTase) or geranylgeranyltransferase I (GGTase I), and allows small GTPases to bind to the plasma membrane. This binding is required for participation in signal transduction pathways that regulate cell proliferation and survival, such as the Ras/MEK/ERK and PI3K/Akt pathways.

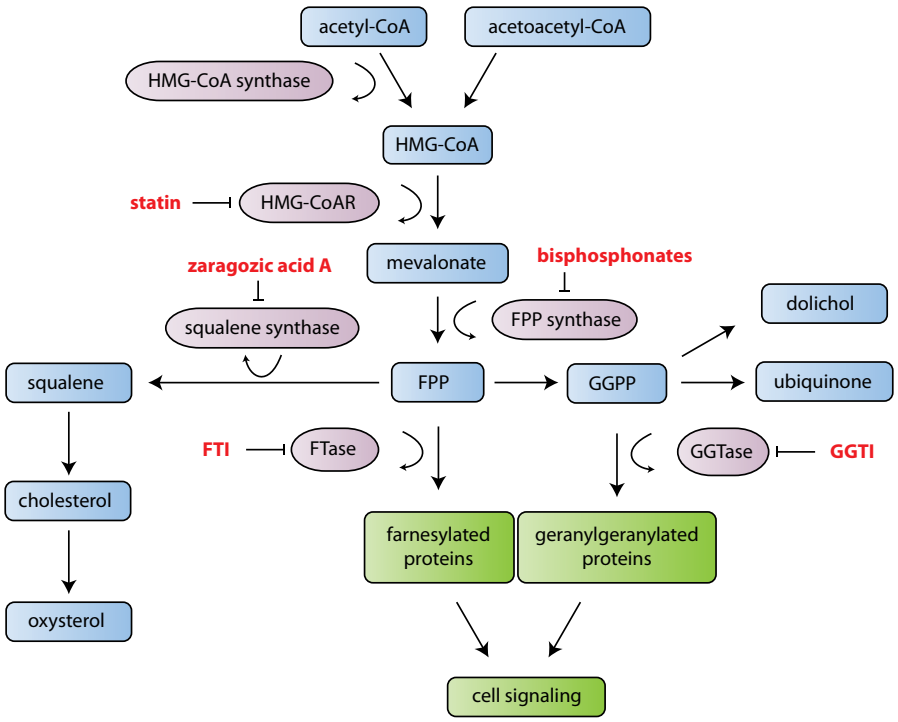

Figure 2. Schematic representation of the mevalonate pathway. HMG-CoAR: 3-hydroxy-3-methyl-glutaryl-CoA reductase; FPP: farnesyl pyrophosphate; FTase: farnesyltransferase; FTI: FTase inhibitor; GGPP: geranylgeranyl pyrophosphate; GGTase: geranylgeranyltransferase; GGTI: GGTase inhibitor.

In the mevalonate pathway HMG-CoAR is the rate-controlling enzyme that converts HMG-CoA to mevalonate. This enzyme is highly expressed in AML[12,13]. Cellular cholesterol levels regulate the expression of HMG-CoAR in normal cells, but AML cells display a deficient feedback inhibition of HMG-CoAR[37], which is likely the cause of the elevated HMG-CoAR expression. Complementary to these data, ectopic HMG-CoAR expression in murine bone marrow increases myeloid colony formation as assessed by methyl cellulose assays[38]. In addition, a meta-analysis of 865 patients retrieved from six primary patient microarray data sets showed that high HMG-CoAR mRNA levels, as well as mRNA levels of additional mevalonate pathway genes, are associated with poor prognosis in breast cancer patients[38]. Together, these findings indicate a role for HMG-CoAR and the downstream mevalonate pathway in the promotion of (leukemic) transformation, but the mechanism of action is still enigmatic.

ABCA1 and ABCG1 are thought to play a role in hematopoiesis. A first indication was the finding that ABCA1 and ABCG1 are highly expressed in primitive normal and leukemic hematopoietic cells[39,40], and that especially ABCG1 is strongly downregulated upon maturation of these primitive cell fractions in mice (personal communication with A. Gerrits, G. de Haan) and humans[39,40]. More recently, the relevance of these two proteins in hematopoiesis has been demonstrated[14]. *Abca1/Abcg1* double KO mice that display an impaired cholesterol efflux showed increased proliferation of myeloid cells, even after transplantation into wild type recipients. Moreover, stem cell and progenitor cell fractions were enlarged in bone marrow of these double KO mice, which could be explained by increased cyclic activity of the bone marrow cells[14]. These effects could be attributed to an increased lipid raft formation[14]. As a result of this, increased cell-surface expression of the common β subunit of the IL-3/GM-CSF receptor was observed, causing stronger proliferative responses upon IL-3 and GM-CSF exposure. This, in turn, lead to increased downstream Ras/ERK signaling and elevated levels of PU.1, a transcription factor that promotes myeloid lineage development, and cyclinD1 in double KO mice[14]. No hematopoietic phenotype was observed when either *Abca1* or *Abcg1* alone were deleted[14], which may be due to functional redundancy of the transporters, as ABCA1 mRNA is increased in *Abcg1* KO mice (K. van der Weide, unpublished data). Surprisingly, in contrast to *Abca1/Abcg1* double KO mice, *Lxr* KO mice do not exhibit expansion of myeloid cells[41], and their stem cell and progenitor frequencies are similar to those of wild type mice (K. van der Weide, unpublished data). This can be explained by the fact that *Lxr*-deficient hematopoietic cells display an increased expression of ABCA1 and ABCG1 despite the absence of their upstream regulator LXR[42].

Many GTPases are known to require isoprenylation by FPP (farnesylation) or GGPP (geranylgeranylation) for activation, and this list is still growing[43,44]. Among these proteins, Ras and Rho are the most intensively studied. N-Ras and K-Ras can be

either farnesylated or geranylgeranylated, whereas H-Ras can only be farnesylated[45]. Constitutive activation of Ras is implicated in the malignant growth of hematopoietic cells. Activating mutations, predominantly in *N-RAS* and less often in *K-RAS*, are found in 15-30% of AML cases[46,47]. Ras activation leads to the subsequent activation of the Raf/MEK/ERK signaling pathway and/or the PI3K/Akt pathway. Both pathways are targets for therapeutic intervention in leukemia[48,49]. Members of the Rho-protein family are generally geranylgeranylated[50]. Rho GTPase family members, such as Rac and CDC42, are essential for interaction of hematopoietic stem and progenitor cells with the bone marrow microenvironment, for stem cell mobilization, and for their proliferation and survival[51]. Deregulation of Rho GTPases is associated with hematological diseases, including AML[52,53].

The mevalonate pathway as a therapeutic target: preclinical data with statins

Statins inhibit HMG-CoAR activity, which leads to inhibition of cholesterol synthesis, but also to a decreased production of isoprenoids. This results *in vitro* in cell cycle arrest[17,55], apoptosis[15,56-58] and cell differentiation[55,59] in AML. The only study in SCID mice showed indeed decreased proliferation of injected AML HL60 cells upon statin treatment[60]. Whether this also holds true for primary patient AML cells is unclear. HL60 is one of the most statin-sensitive AML cell lines[16,56], whereas primary AML cells require an at least 20-fold higher statin concentration for comparable inhibitory effects[61].

Several statins are available for clinical use, which are either fermentation-derived (simvastatin, lovastatin, pravastatin, mevastatin) or synthetically produced (atorvastatin, cerivastatin, fluvastatin, pitavastatin, rosuvastatin). Studies with these different statins resulted in varying results. Some *in vitro* effects are only seen with the natural statins[62], which may be due to their chemical structure and binding affinities for HMG-CoAR[63]. In addition, cerivastatin appeared to be at least ten times more potent in AML cells than other statins[64], and simvastatin is more effective in myeloma cells than lovastatin or atorvastatin[65]. Therefore, data of studies employing different statins should be carefully interpreted.

Being the direct target of statins, it is not surprising that HMG-CoAR is thought to play a key role in the statin-induced cytostatic and cytotoxic effects in tumor cells. Addition of mevalonate, the direct downstream product of HMG-CoAR, prevents *in vitro* statin-induced effects[15,16,57,58,66]. Moreover, the statin-induced response in AML is inversely correlated with expression levels of HMG-CoAR[58], although this could not be confirmed by others[16].

Different biochemical mechanisms of statin-induced effects have been proposed. Both statins and zaragozic A, a cholesterol synthesis inhibitor downstream from FPP (Figure 2), sensitized AML cells to chemotherapy[9,29,67]. In contrast, by mechanistically

uncoupling cholesterol synthesis from isoprenoid synthesis, others have demonstrated a more prominent role for isoprenylation, rather than cholesterol synthesis in statin-induced cell death of AML cells[15,16,18,68]. With a similar setup, it was shown that leukemia cells are more dependent on geranylgeranylation than on farnesylation for survival[15,17,65]. However, the reduced ability of FPP to prevent simvastatin-induced cytotoxicity may be due to the additional production of squalene from FPP. Therefore, it is not entirely justified to conclude that FPP is less relevant than GGPP for statin-induced cytotoxic effects. Indeed, in AML cells both farnesylation and geranylgeranylation were shown to play an active role in cell survival[16]. In addition, in multiple myeloma, FPP synthase and GGPP synthase are upregulated upon lovastatin treatment in statin-insensitive cells but not in sensitive cells[69]. These findings suggest a role for both FPP and GGPP in statin-induced cytotoxicity.

Inhibition of isoprenoid synthesis by statins can result in inhibition of Rac-1[70,71], Ras[18,67,71,72], and Rho activation[73-75]. As these small GTPases are relevant for cell growth and survival, it was thought that the antitumor effects of statins are dependent on inhibition of Ras function[60,67]. However, patient AML cells bearing an activating Ras-mutation were usually not more sensitive to *in vitro* statin treatment than cells without this mutation[60,67]. In addition, in NIH-3T3 cells transformed with myristylated Ras (myr-Ras), and therefore independent of isoprenylation for appropriate membrane localization and function, are equally sensitive to statin-induced growth inhibition as cells transfected with wild type Ras[76]. So, statin-induced effects are not solely dependent on Ras function and may also involve other isoprenylated proteins[43,44].

In patients, plasma concentrations of up to 3.9 μM lovastatin can be achieved with a lovastatin dosage of 25 mg/kg/day[77]. In many of the *in vitro* studies, statin concentrations applied are much higher, starting at 20 μM[18,29,56,61,78,79]. It is therefore important to determine the effects *in vivo* at low, nontoxic concentrations that can be clinically achieved. It appeared that inhibition of farnesylation takes place at simvastatin concentrations of 5-10 μM in several relevant cell lines. Even higher concentrations were required in AML patient-derived cells. Yet, inhibition of geranylgeranylation could be realized at concentrations of less than 1 μM in both AML cell lines and AML patient cells (this thesis, *chapter 6*)[16]. This may, given the maximal achievable plasma concentration, have implications for the *in vivo* effects of statins.

An alternative manner by which statins can affect AML cell growth is by affecting the *in vivo* microenvironment. This microenvironment consists of an endosteal niche, composed of osteoblasts, osteoclasts and stromal cells, and a vascular niche, containing sinusoid endothelial cells. Osteoblasts can interact with normal and leukemic hematopoietic stem cells, thereby preserving their stem cell phenotype. This causes a release of hematopoietic cytokines and growth factors, and a subsequent activation of

signal transduction pathways[80]. Statins induce differentiation of osteoblasts and inhibit proliferation of bone marrow stromal cells at low concentrations (0.01-1 μM)[81-84]. This may disrupt interaction of (leukemic) bone marrow cells with their microenvironment. Thus, the suppressive effects of statins are not only directed to the malignant cell, but can also indirectly affect the leukemic cells by modulating the microenvironment. However, the effects of statins on the bone marrow microenvironment and cytokine production by AML cells are as yet insufficiently established.

Clinical application of statins

The first clinical trial with statins in cancer patients, not involving AML patients, showed that lovastatin administered at a dosage of 25 mg/kg/day for 7 consecutive days was well tolerated with only minimal dose-related side effects[77]. This dosage is about 50-fold higher than administered to hypercholesterolemic patients[85,86], and resulted in plasma concentrations of 0.1 to 3.9 μM[77]. However, there was a marked interpatient variability independent of the administered dosage[77]. To evaluate the safety and efficacy of lovastatin in AML patients, a trial was conducted using a dosage of 10-20 mg/kg/day for 2 weeks. This study was prematurely closed because of drug-related side effects[63]. In a single case study in a patients with relapsed AML, it was shown that a low dosage lovastatin (2 mg/kg/day) could be given for a prolonged period of time (54 days). In addition, AML blast cell counts were decreased, which persisted for 3 months after cessation of therapy[87]. A phase 1 study conducted in 37 AML patients showed that pravastatin (up to ~25 mg/kg/day) followed by idarubicin and cytarabine treatment could safely be given. Although the small number of patients and non-randomized nature of the trial impedes to draw solid conclusions on the benefit of the treatment, some encouraging response rates were observed[30]. For example, the CR rate of newly diagnosed patients with unfavorable cytogenetics and first relapse patients was higher than the historical CR rates.

Limitations of statin use

The use of statins as complementary antileukemic treatment of AML patients will not be effective in all patients. Therefore, it is of interest to select biomarkers that can help to predict which patients may benefit from statin treatment. *In vitro* studies have already shown differences in sensitivities within AML cell lines and primary AML samples[16,56,61,78]. These different studies showed consistent results regarding the proportion of statin sensitive patient samples: about half of the patient AML samples required less than 100 μM statin to obtain 50% cytotoxicity[56,61,78]. Interestingly, when examining separately primitive $CD34^+(CD38^-)$ AML cells and more mature $CD34^-$ AML cells, the heterogeneity in response appeared to reside in the $CD34^+(CD38^-)$ fraction[61,78]. $CD34^+$

and CD34$^-$ cells are essentially different regarding long term proliferation capacity and susceptibility to apoptosis[88,89] and CD34$^+$ (AML) cells display higher expression levels of cholesterol metabolism genes[39,40]. Therefore, it is of interest to use primitive CD34$^+$ AML cells in the search for predictive biomarkers.

Recently, we and others aimed to get insight in the mechanisms underlying the differences in response to statins. In multiple myeloma, statin-resistant cell lines expressed higher HMG-COAR as well as HMG-COA synthase 1 mRNA and protein levels, which were further upregulated upon statin treatment. In contrast, statin-sensitive myeloma cells did not show increased expression of these genes[69]. Also AML cells express higher levels of HMG-COAR compared with their normal counterparts[13], and are more sensitive to statins than normal hematopoietic cells[56]. However, basal HMG-COAR expression was not necessarily higher in statin-insensitive AML cells compared with statin-sensitive AML cells, nor was statin-mediated induction of expression of HMG-COAR mRNA[16]. This suggests that the mechanism behind heterogeneity in statin response does not solely exist at the level of HMG-COAR expression. Alternative splicing of HMG-COAR, which renders the protein inactive, can also induce intra-individual differences in response to statins[90]. However, this splice variant is expressed considerably lower than the full-length protein[69], and the variation in statin-induced HMCGR-D13 expression only accounts for 6-15% of the variation in statin response[91].

Studies in AML suggest that the cause of heterogeneity in statin response lies within the isoprenylation pathway[16,92]. In AML cell lines with different sensitivities to simvastatin, especially in the sensitive cell lines statin-induced cytotoxicity could be prevented by FPP or GGPP. In addition, sensitive cell lines display a higher degree of isoprenylation inhibition of proteins like Ras as well as inhibition of phosphorylation of the Ras-target ERK upon statin treatment than insensitive cell lines[16]. The exact players are however as yet unknown, and it is likely that isoprenylated proteins other than Ras contribute to the differences. The ratio of the expression of *RASGRP1* and *APTX* is a predictor for the response of AML patients to treatment with tipifarnib, a farnesyltransferase inhibitor[93]. As sensitivity to tipifarnib corresponds with sensitivity to statins in AML cells[16], the mechanism of resistance to these compounds may overlap, so assessing the ability of the *RASGRP1:APTX* ratio to predict statin sensitivity might be of interest.

Compared to *in vitro* studies, statin treatment in AML patients adds additional factors that cause different responses in AML patients. For example, activity of CYP3A4, the enzyme that converts most of the statins into their active form, varies among patients due to genetical differences or extrinsic factors, e.g., the use of other drugs and diet composition[94]. Besides CYP3A4, there are many other gene products responsible for varying pharmacokinetics and pharmacodynamics of statins that cause variation

in plasma statin levels and their efficacy, among which are proteins that affect intestinal cholesterol absorption (ABCG5, ABCG8, APOE), cholesterol production (HMG-COAR), and lipoprotein catabolism (APOB, LDLR)[86]. There are nowadays over 30 genes known as potential determinants of the cholesterol-lowering capacity of statins[95]. Another hurdle for the application of statins in AML patients is that, due to an extensive first-pass metabolism by the liver, the plasma levels reached are too low to induce cytotoxic effects, even with high dosages of the drug[96]. Interestingly, this first-pass effect may explain the finding in a mouse model where simvastatin affected cholesterol metabolism gene expression in the liver, but not in bone marrow or peripheral blood cells (this thesis, *chapter 6*). The plasma concentration that can be achieved with high-dose (25 mg/kg/day) lovastatin, namely 3.9 μM[77], is insufficient to induce cytotoxic effects or changes in cholesterol metabolism in cultured patient AML cells (this thesis, *chapter 6*). Moreover, in rats the concentration of orally administered lovastatin was in bone marrow 10-fold lower than in plasma[97]. Still, these concentrations may be high enough to induce inhibition of geranylgeranylation, as observed for concentrations of as low as 0.2 μM in cell lines as well as primary normal or AML bone marrow cells (this thesis, *chapter 6*).

To increase statin plasma levels, higher dosages should be administered, which may lead to major side effects such as myopathy. Ubiquinone supplementation has been used to prevent myopathy, as it does not abrogate the potential therapeutic effects in AML[98]. In a phase 1 study evaluating the effects of high-dose lovastatin, ubiquinone supplementation reduced the severity, not the incidence, of myopathy[77], but in general the evidence demonstrating the efficacy of ubiquinone is thin[99].

Another way to increase plasma statin levels would be by alternative administration routes to bypass first pass clearance by the liver, like intravenous administration. Unfortunately, intravenous applicable formulations of statins are not available[100]. There are synthetic statins available with a longer half-life, such as rosuvastatin and atorvastatin, which may also contribute to higher plasma levels. Regretfully, these statins are likely less effective in AML cells, because their hydrophilic nature impedes their passive diffusion across the cell membrane[94].

Statins are administered in most clinical trials for 7 days. However, as statins can induce toxicity in AML cells already within 72 hours[101], a shorter treatment period can be of interest. In a trial in patients with advanced malignancies, high dosages lovastatin (~50 mg/kg/day) were administered orally every 6 hours for 4 days. Peak plasma levels of 12 μM were reached and no drug-related toxicity was observed[102]. This indicates that higher dosages applied for a shorter period of time result in higher statin plasma levels without inducing side-effects.

Combination treatment with statins

Although statins will not be used as single agent in AML treatment, these drugs are of potential interest in combination with standard antileukemic agents. Several statins increase the cytotoxicity of cytarabine[29,61,66,67,103], daunorubicin[61], paclitaxel[104], and all-trans retinoic acid[55] in cultured AML cells, and sensitize these cells to radiation[29]. Suggested mechanisms underlying these synergistic interactions are inhibition of NF-κB[62], cell cycle arrest in G1[105], attenuation of cholesterol increments[29], and abrogation of cell adhesion-mediated drug resistance by inhibition of Rho-activity[106].

A mevalonate-independent mechanism of statins in sensitization to chemotherapy is due to interference with P-glycoprotein (P-gp) action[107]. P-gp, also called ABCB1, is a member of the ABC transporter family. It is a drug efflux pump for xenobiotics including chemotherapeutics, which can result in resistance to chemotherapy. Lovastatin directly binds to P-gp and thus prevents P-gp-mediated drug efflux in multidrug resistant tumor cells[107]. In contrast to fungal-derived statins (e.g., atorvastatin, rosuvastatin), synthetic statins with a different chemical structure do not block P-gp[108]. Other researchers show a mevalonate-dependent inhibition of P-gp[109]. This is possibly mediated by depletion of dolichol, a mevalonate derivative essential for glycosylation of P-gp[109]. In addition, P-gp expression can be downregulated by either statins or deprivation of LDL cholesterol in KG1a AML cells[110]. P-gp was also downregulated in peripheral blood mononuclear cells upon a 4-week atorvastatin treatment of hypercholesterolemia patients[111]. This provides another mechanism by which statins may overcome chemoresistance, although recent studies with specific P-gp inhibitors added to chemotherapy did not improve the clinical outcome in AML patients[112].

The (pre)clinical benefits of combining chemotherapy with statins are not yet apparent. Out of 7 AML patients treated with high-dose simvastatin the cultured AML cells of two patients were more sensitive to chemotherapy compared with the cells from the same patients before treatment (this thesis, *chapter 6*). The only clinical study in which a statin, pravastatin, was combined with chemotherapy (idarubicin/high-dose cytarabine) did not allow to draw valid conclusions about the antitumor efficacy of the combination treatment compared with single treatment due to its phase 1 nature. However, it did show that a high dosage pravastatin can be safely administered in combination with chemotherapy[30]. Currently, phase 2 trials are ongoing to assess the efficacy of combined treatment with statins and chemotherapy.

Isoprenylation inhibitors as alternatives for statins

Farnesyltransferase inhibitors (FTIs) act indirectly on the mevalonate pathway by blocking the transfer of farnesyl isoprenoid groups to small GTPases. FTIs have been developed to specifically target oncogenic Ras proteins. These proteins are frequently

mutated in human cancers. In addition, there are several additional targets for FTIs such as RhoB, nuclear lamins and the centromere proteins CENP-E and -F[113,114]. FTIs, including tipifarnib, inhibit the proliferation of leukemic cells[113] in preclinical studies. In the initial phase 1 clinical trial, refractory and relapsed AML patients received 100-1200 mg tipifarnib twice daily for 21 consecutive days during 4 weeks. There was no toxicity, and an interesting overall response rate of 29%, including 2 CRs (6%), was observed[115]. In a subsequent phase 2 study in 158 previously untreated older adults with poor-risk AML, the overall response rate was 23% with 14% CR. Yet, serious side effects were observed in 47% of the patients[116]. A parallel and comparable study in 252 patients resulted in an 11% overall response rate and 4% CRs[117]. Overall, these results do not advocate the use of tipifarnib as a single agent. The combined use of tipifarnib with cytarabine and daunorubicin was well tolerated up to a tipifarnib dosage of 600 mg twice daily[118]. However, in a phase 3 study in which tipifarnib treatment was compared with best supportive care (including hydroxyurea) in elderly patients with AML, there was no survival benefit for the tipifarnib arm[119]. In addition, a phase 1-2 study in 95 AML patients showed for the combination tipifarnib, cytarabine and idarubicin a response rate similar to cytarabine and idarubicin alone[120].

A potential explanation for the limited responses on tipifarnib is that, although tipifarnib inhibits farnesylation of Ras proteins, K-RAS and N-RAS can undergo alternative prenylation by geranylgeranyltransferase (GGT) in FTI-treated cells[121]. Therefore, it may be of potential interest to combine FTI treatment with GGT inhibitors (GGTIs) or statins. Indeed, *in vitro* cotreatment of simvastatin and tipifarnib results in additive cytotoxic effects in primary AML cells. In addition, phosphorylation of the Ras target ERK is inhibited by the combination, whereas either agent alone does not alter the phosphorylation status of ERK[78]. Studies in mice and AML patients indicate that inhibition of geranylgeranylation occurs at clinically relevant statin concentrations (this thesis, *chapter 6*). GGTIs are more specific and selective than statins and do indeed act synergistically in multiple myeloma cells [122,123]. However, combining an FTI with a GGTI seems clinically not feasible as GGTI treatment appeared lethal in a mouse study[124].

Bisphosphonates (BPs) are used to treat metabolic bone disease, as they inhibit osteoclast-mediated bone resorption and bone metastases. In addition, anti-cancer properties have been described (reviewed by Green and Lipton[125]). BPs inhibit farnesylpyrophosphate synthase, which results in a decreased production of both FPP and GGPP. Application of a standard dosage (4 mg/15 minutes IV) of the BP zoledronic acid results in maximal plasma concentrations of 1 μM[126], which is much lower than the concentration required for cytotoxic effects in AML cells[127]. However, the concentration of BPs that can be achieved in bone marrow is much higher than in plasma[128,129], so clinical testing in the setting of AML may be worthwhile.

Potential role of statins in GVHD in allogeneic bone marrow transplantation

Recently, an additional role of statins in AML was recognized in bone marrow transplantation. Allogeneic hematopoietic stem cell transplantation coincides with morbidity and mortality due to graft-versus-host disease (GVHD). Statins affect the immune responses through a variety of mechanisms (reviewed by Arnaud et al.[130]), which can be ascribed to their inhibitory effects on isoprenylation and subsequent activation of small GTPases[131-134]. However, statins can also inhibit lymphocyte function-associated antigen-1 (LFA-1), involved in T cell activation through binding to an allosteric L-site of LFA-1. Statins were shown to protect against GVHD in a mouse model[135,136] and a retrospective study in 67 patients of whom 10 took statins suggested a lower incidence of acute GVHD for statin users, while graft-versus-leukemia was preserved[137]. In addition, a retrospective analysis in patients with hematopoietic malignancies who underwent allogeneic hematopoietic stem cell transplantation demonstrated an association between statin use of their donor and a decreased risk of grade 3-4 GVHD in the patients[138]. These retrospective studies are challenging, but, given their retrospective nature, inconclusive. Therefore, prospective clinical studies, such as the ongoing trial with atorvastatin for acute GVHD prophylaxis in patients undergoing allogeneic bone marrow transplantation (http://www.clinicaltrials.gov: NCT01175148), are of interest. So, by lowering the incidence of GVHD, low-dose statins offer another therapeutic implication in the treatment of AML.

Concluding remarks

AML cells display an aberrant cholesterol metabolism, which provoked the application of statins in the treatment of the disease. It is currently known that statins, apart from suppressing cholesterol synthesis, exert additional cellular effects such as the inhibition of isoprenoid production. Blockade of isoprenoid synthesis, rather than of cholesterol synthesis, appears to be mainly responsible for the observed antitumor effects of statins. The initial enthusiasm for statin use in AML patients was halted by results of clinical studies, in which the activity of the single agent or the activity when combined with chemotherapy was not evident. A hurdle in this respect is that the plasma levels achieved, even at high dosages of these drugs, are insufficient to induce cytotoxic effects. However, statins do inhibit geranylgeranylation *in vivo,* and it is important to identify more relevant therapeutic targets of statins. The clinical relevance of solely inhibiting geranylgeranylation is still unclear, especially since alternative farnesylation can overcome the potentially beneficial effects. Therefore, combination with other agents such as chemotherapy or FTIs may be of interest, even more because combination treatment could require lower plasma levels of the agents. *In vitro* data indicate that the cells of a subset of AML patients certainly show a response to statin treatment,

whereas others appear to remain unaffected. When the mechanisms underlying the heterogeneity in response of AML cells are known, this could lead to biomarkers that can predict which patients will benefit from statin treatment. The heterogeneity in AML response to statins resides in the isoprenylation pathway, but more research is required to pinpoint the exact mechanisms of action and corresponding molecular markers. Furthermore, it is also important to focus on the indirect effects of achievable concentrations of the clinically proven and safe statins in humans that affect leukemic cell survival as well, which include a decreased incidence of GVHD and effects on the bone marrow microenvironment.

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Variability in responsiveness to lovastatin of the primitive CD34$^+$ AML subfraction compared to normal CD34$^+$ cells

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ABSTRACT

In the present study, we questioned whether the cholesterol synthesis inhibitor lovastatin potentiates the cytotoxicity of chemotherapeutic agents in the primitive CD34$^+$ subpopulation of acute myeloid leukemia (AML) cells. AML mononuclear cells (n=17) were sorted in CD34$^+$ and CD34$^-$ fractions and compared to normal CD34$^{+/-}$ cells (n=7). The percentage of surviving cells upon exposure to lovastatin (25-100 μM) and/or chemotherapeutics (cytarabine or daunorubicin) was determined with a luminescent cell viability assay. The results demonstrate that the primitive CD34$^+$ subpopulation of normal and AML cells displayed a higher sensitivity to lovastatin than the more mature CD34$^-$ subpopulation. The combination of lovastatin and chemotherapeutics resulted in a more pronounced inhibitory effect on both subpopulations. In contrast to the homogeneous results in normal CD34$^+$ cells, a distinct heterogeneity in lovastatin sensitivity was found in AML samples. Therefore, a group of normal (n=11) and abnormal (n=6) responders were identified based on a reduced or increased cell survival compared to normal CD34$^+$ cells. This distinction was not only observed in the CD34$^+$ AML subfraction but also in CD34$^+$CD38$^-$ AML cells. In the abnormal responder group, 50% of patients presented with unfavorable cytogenetics and significant higher peripheral blast cell counts, which coincided with poor treatment results. In summary, the findings indicate that the primitive subfraction of CD34$^+$ AML cells is in the majority of cases affected by lovastatin treatment, which is potentiated when combined with chemotherapeutics. Heterogeneity of the response observed in AML patients allowed identification of a subgroup with poor prognosis.

INTRODUCTION

Acute myeloid leukemia (AML) is characterized by accumulation of immature myeloid cells in the bone marrow. These malignant cells are usually hierarchically structured, similar to the normal hematopoietic system. We have reported that, according to this hierarchal structure, ATP-binding cassette (ABC) transporters are expressed in a differentiation-dependent manner[1]. Specifically, in primitive $CD34^+CD38^-$ cells, a high expression of a number of ABC transporters genes was noticed in comparison to the more differentiated $CD34^+CD38^+$ cells. Notably, in the leukemic counterpart, a much more heterogeneous pattern of ABC transporter gene expression was observed[1]. Recently, de Grouw et al. published expression data for all 45 ABC transporters in AML cells[2]. They found 22 ABC transporters to be lower expressed in the $CD34^+CD38^+$ subpopulation compared to the $CD34^+CD38^-$ subpopulation. In addition to the two major cholesterol efflux transporters (ABCA1 and ABCG1), two genes critical in cholesterol metabolism ((3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase and low-density lipoprotein receptor (LDLR)) were highly expressed in normal $CD34^+CD38^-$ cells, indicative for an active cholesterol metabolism[1,3].

In AML cells, an aberrant cellular cholesterol metabolism has been demonstrated, including a higher LDLR activity[4]. Interestingly, it was recently shown *in vitro* that cells isolated from a subgroup of AML patients demonstrated in their leukemic cells 'an acute cholesterol response', i.e., a rapid increase in cellular cholesterol content in response to cytotoxic agents[5]. These AML cells were further characterized by an increased chemosensitivity upon blocking this acute cholesterol response by pretreatment of cells with a statin[4]. Statins act as competitive inhibitors of the enzyme HMG-CoA reductase and hence block cellular cholesterol synthesis. Consequently, a role for these cholesterol synthesis inhibitors to improve standard antileukemic treatment has been suggested[4,6-10]. In the present study, we focused on a subpopulation of AML cells ($CD34^+$), i.e., cells that have more in common with the more primitive leukemic progenitor/stem cell compartment[11], and questioned whether this subpopulation of cells is especially prone to the effects of lovastatin and chemotherapeutic agents. Our data demonstrate a higher sensitivity of the primitive $CD34^+$ subpopulation for lovastatin compared to the more mature $CD34^-$ subpopulation of normal as well as AML cells. Heterogeneity of the response observed in AML patients allowed identification of a subgroup with poor prognosis.

MATERIALS AND METHODS

Normal CD34$^{+/-}$ hematopoietic cells

Normal mobilized peripheral blood cells were collected either from healthy donors or from patients awaiting autologous stem cell transplantation, undergoing granulocyte colony-stimulating factor (G-CSF) treatment, in accordance with institutional guidelines. CD34$^+$, CD34$^-$14$^+$15$^+$ (CD34$^-$), CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ cells were obtained by means of the MoFlo flow cytometer (Dako Colorado Inc., Fort Collins, CO).

Patients

After informed consent, bone marrow or peripheral blood cells were collected of AML patients at diagnosis. The Human Subject Review Board of the University Medical Center Groningen (the Netherlands) approved the protocol. Patients were classified according to the WHO classification[12]. Mononuclear cells were enriched by Ficoll-Isopaque (Nycomed, Oslo, Norway) density gradient centrifugation and cryopreserved in RPMI 1640 medium (BioWhittaker, Brussels, Belgium) supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT) and 10% dimethyl sulfoxide (Merck, Amsterdam, the Netherlands), and stored at –196°C as described[13].

Materials

Mevinolin (L-154, 803-00G17) in the lactone form was obtained from Merck, Sharp & Dohme Research Laboratories (Rahway, NJ, USA), converted to its sodium salt, and a stock solution of mevinolin (lovastatin) at a concentration of 4 mg/mL was prepared as previously described. The cytotoxic agents daunorubicin (Aventis Pharma BV, Hoevelaken, the Netherlands) and cytarabine (Mayne Pharma (Benelux) SA-NV, Brussels, Belgium) were used.

Flow cytometric sorting

The normal and AML samples were incubated with a fluorescein isothiocyanate (FITC)-conjugated antibody against CD34 (Becton Dickinson, San Jose, CA, USA) and a phycoerytrin (PE)-conjugated antibody against CD14 and CD15 or CD38. Sorting was done using a MoFlo flow cytometer. Erythrocytes and dead cells were excluded from analysis by gating on forward and sideward light scatter. For RNA extraction, at least 3×10^4 CD34$^+$ and 3×10^4 CD34$^-$CD14$^+$CD15$^+$ cells were sorted. No difference existed in average CD34 expression between normal and leukemic cells mean fluorescence intensity.

Luminescent cell viability assay and quantitative PCR

A cell viability assay was used for determining the number of viable cells in culture based on quantitation of the ATP present, which signals the presence of metabolically active cells. The amount of ATP is directly proportional to the number of cells present in the culture. The ATP measurement was carried out according to the manufacturer's instructions using the Cell Titer-Glo Luminescent cell viability assay (Promega, Madison, WI, USA). We prepared 96-well plates with 100 µL RPMI 1640 medium supplemented with 10% FCS and sorted by the MoFlo flow cytometer, either 10^4 $CD34^+$ cells or $CD34^-CD14^+CD15^+$ cells per well. The wells were incubated with different concentrations of lovastatin (25, 50, and 100 µM) with and without different concentrations of either daunorubicin (0.01-0.5 µM) or cytarabine (0.0001-0.1 mg/mL) using easy load pipet tips (Greiner Bio-One, Alphen aan den Rijn, the Netherlands) and analyzed after 20 hours. All experiments were done in duplicate. No difference existed in baseline average and median ATP levels between $CD34^+$ and $CD34^-$ cells. RNA extraction, CDNA synthesis, and quantitative polymerase chain reaction were done as described before[13].

Colony-forming cell assay

One thousand normal or AML $CD34^+$ cells were sorted into Iscove's Modified Dulbecco's Media (IMDM) containing 10% FCS, 20 ng/mL interleukin-3 (IL-3), 100 ng/mL c-Kit ligand, 100 ng/mL Flt-3 ligand and 100 ng/mL thrombopoietin as described. Twenty-four hours after treatment with different concentrations lovastatin, cytarabine and daunorubicin, cells were plated into MethoCult H4230 (StemCell Technologies, Vancouver, Canada) supplemented with 20% IMDM, 20 ng/mL IL-3, 20 ng/mL G-CSF, 20 ng/mL IL-6, 20 ng/mL c-Kit ligand and 1 U/mL EPO (Cilag: Eprex, Brussels, Belgium).

Statistical analysis

Friedman's and Mann-Whitney's nonparametric tests were done to calculate significant differences, p-values of <0.05. SPSS 14.0 statistical software (SPSS Inc. 2005) was used to analyze the data. Additionally, we looked for additive and synergistic effects of the combination treatment of lovastatin and chemotherapeutics. Additivity was defined as an increased inhibitory effect on cell survival of the combination exposure compared to the single exposure of lovastatin or chemotherapeutics. Synergism was defined by two individual agents (lovastatin and chemotherapeutics) acting together and creating an inhibitory effect on cell survival greater than predicted by knowing only the separate inhibitory effects of the individual agents.

Table 1. Clinical and cellular characteristics of AML patients

AML	Age (yr)	Leuko[a] (×10⁹/L)	FAB Class.	Cytogenetics[b]	Treatment[c] (it/pt)	Clinical response[d]	EFS[e] (mo)	OS[f]	CD34+[g] (%)	Response to lova[h]	Risk-group stratification[i]
1	59	128	M1	N	it	CR	18	1	24	N	Intermediate
2	68	4	M5b	N	it	CR	15	0	64	N	Intermediate
3	51	33	M1	N	it	CR	37	1	63	N	Intermediate
4	73	6	M2	N	it	CR	12	1	28	N	Intermediate
5	79	96	M5b	N	pt	NR	-	0	15	N	Intermediate
6	19	10	M2	N	it	CR	13	1	23	N	Intermediate
7	43	24	M5	N	it	CR	1	0	28	N	Intermediate
8	64	11	M5	inv(16)	it	CR	8	1	20	N	Good
9	48	16	M1	N	it	CR	8	0	17	N	Intermediate
10	49	50	M4/M5	N	it	CR	2.5	0	7	N	Intermediate
11	55	2	M2	N	it	CR	15	0	56	N	Intermediate
12	44	66	M5	3q-,5q-, +8	it	NE[tox]	0	0	8	A	Poor
13	54	48	M5a	N	it	NE[tox]	0	0	85	A	Intermediate
14	67	63	M1	t(6;9), trisomy 13	it	NR	0	0	35	A	Poor
15	19	102	M5	inv(16)	it	CR	21+	1	60	A	Intermediate
16	42	200	M1	inv(3q), 7-, 10-	it	NR	0	0	87	A	Poor
17	38	96	M1	N	pt	NR	-	0	92	A	Intermediate

[a]Leukocytes at presentation; [b]Cytogenetics: N: normal; inv: inversion; [c]Treatment: it: intensive treatment; pt: palliative treatment; [d]Clinical response: CR: Complete remission; NR: non-responder; NE[tox]: not evaluable due to treatment related toxicity; [e]For event-free survival (EFS), a plus following the value indicates still no relapse as at September 12, 2007; [f]OS: Overall survival: 0: death and 1: alive; [g]Percentage $CD34^+$ cells in the AML mononuclear cell fraction; [h]Response of $CD34^+$ cells to lovastatin. N: Normal responders were defined as AML $CD34^+$ cells demonstrating a similar effect on cell survival as normal $CD34^+$ cells. A: Abnormal responders were defined as AML $CD34^+$ showing a dissimilar effect on cell survival to normal $CD34^+$ cells; [i]Risk group stratification is based on (un)favorable cytogenetics combined with peripheral blood blast cell counts.

RESULTS

Patient characteristics

The AML patients (n=17) studied, median age of 51 (range 19-79), were classified according to the WHO classification: AML with cytogenetic abnormalities (n=5), AML with prior dysplastic syndrome (n=2), and the additional group consisting of M1 (n=4), M2 (n=3) and M4/M5 (n=3). The clinical and cellular characteristics of the patients are shown in Table 1. The median peripheral blast cell count at diagnosis was 48.6×10^9/L (range 2.2-200). The majority of the patients were treated according to ongoing Haemato-Oncology Co-operative Group (HOVON) protocols, i.e., the HOVON Swiss Group for Clinical Cancer Reseach AML-42 study for patients <60 years[14] and the HOVON 43 study for patients >60 years[15]. Palliative treatment was given to patients that were ineligible for intensive chemotherapy (n=2). These patients were treated with 6-mercaptopurine[16]. Eleven patients reached complete remission (CR) on protocol. In six patients, CR was not attained due to progression (n=2) or to treatment related toxicity (n=4). The median event-free survival (EFS) of all patients attaining CR was 13 months (range 1-37).

The CD34⁺ subpopulation of normal and AML hematopoietic cells is more sensitive to lovastatin treatment than the more mature CD34⁻ subpopulation

To evaluate whether differences exist in statin sensitivity between primitive and more differentiated cells, normal $CD34^+$ and $CD34^-14^+15^+$ subfractions (n=7) were sorted from the mononuclear cell fraction from normal peripheral blood stem cells. A clear discrepancy between both fractions was observed upon lovastatin exposure. The $CD34^+$ subpopulation demonstrated a reduction of cellular ATP to 64% at a dose of 50 μM lovastatin compared to 123% in the $CD34^-$ fraction (p=0.008; Figure 1A).

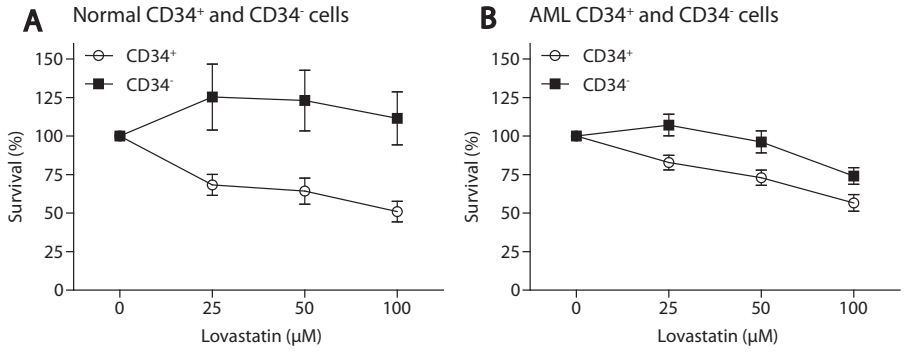

Figure 1. Cell survival of CD34⁺ and CD34⁻ normal (A: n=7) and AML cells (B: n=17) upon incubation with increasing lovastatin concentrations (25-100 μM). SEM are indicated.

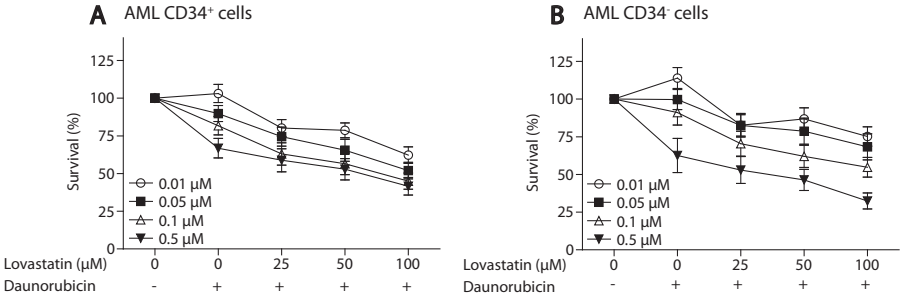

Figure 2. Effects of increasing concentrations of chemotherapeutic drugs on in vitro survival of subpopulations of AML cells. $CD34^+$ (A) and $CD34^-$ (B) AML cells of all patients (n=17) were incubated with lovastatin in combination with variable concentrations of daunorubicin (0.01-0.5 µM). SEM are indicated.

A similar experimental setup was used for the AMLs (n=17). From the total AML cell fraction the $CD34^+$ subfraction was separated from the $CD34^-$ fraction by MoFlo. The percentage of $CD34^+$ cells varied strongly between the different AML samples as depicted in Table 1 (median 41% (range 7%-92%)). Subsequently, the $CD34^+$ and $CD34^-$ fractions were exposed to varying concentrations of lovastatin (25-100 µM) for 20 hours and the effects on cell survival were assessed. As depicted in Figure 1B, a significant difference in sensitivity for lovastatin was observed for the AML $CD34^+$ versus $CD34^-$ fraction. At 50 µM, a median reduction of 30% in survival (range minus 24-54) was observed for the $CD34^+$ cells compared to 12% (range minus 51-42) in the $CD34^-$ fraction (p=0.029). A similar pattern was also observed at 100 µM (43% versus 23%, p=0.029). Subsequently, we compared the AML subpopulations with the normal $CD34^+$ and $CD34^-$ cell fractions and observed a significant increased lovastatin sensitivity of the AML $CD34^-$ fraction compared to the normal the $CD34^-$ subpopulations (p=0.005; Figure 1). This was not noticed for the AML and normal $CD34^+$ cell fractions (p=0.095). Based on the pattern of lovastatin sensitivity of the normal $CD34^+$ cells, two subgroups within the AMLs could be distinguished. Eleven AML $CD34^+$ samples had a response pattern comparable to normal $CD34^+$ cells, whereas six showed a reduced lovastatin sensitivity (Figure 3; Table 1). A comparable pattern was observed for the AML $CD34^-$ fraction.

The combination of lovastatin and chemotherapeutics potentiates cell death

To evaluate whether the cytotoxic effects of two frequently used cytostatic agents, cytarabine and daunorubicin, might be promoted by cotreatment with lovastatin, the AML $CD34^+$ and $CD34^-$ subfractions were incubated with varying concentrations of lovastatin (25, 50, and 100 µM) in the presence or absence of varying concentrations of daunorubicin (0.01-0.5 µM) or cytarabine (0.0001-0.1 mg/mL) for 24 hours. A dose-dependent

decrease in cell survival was seen as depicted in Figure 2, which was augmented by lovastatin treatment. A median survival reduction of 21% (range minus 28-64%) was accomplished by exposing the AML CD34⁺ cells to 0.1 μM daunorubicin, which was 43% (range minus 56-78%) when the CD34⁺ AML cells were exposed to the combination of daunorubicin and 25 μM lovastatin (Figure 2A).

The combined use was slightly more effective in the CD34⁺ subpopulation compared to the CD34⁻ subpopulation (Figure 2B; median 43% versus 36%) but the difference did not reach statistical significance. Comparable results were obtained for

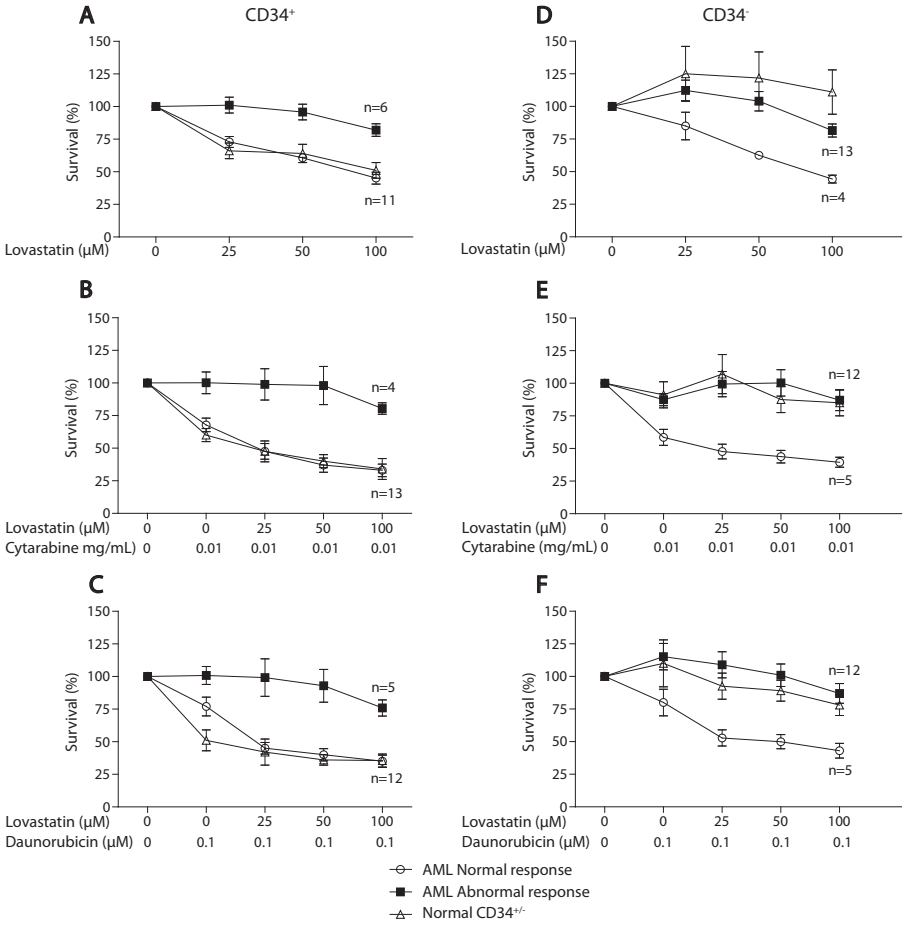

Figure 3. Effects of increasing concentrations of lovastatin in combination with chemotherapeutic drugs on in vitro survival of CD34⁺ (A-C) and CD34⁻ (D-F) normal and AML cells. Three groups are demonstrated in graphs 3A-F. Two AML patient groups (normal responders (n=13) and abnormal responders (n=4); in total: n=17) and a normal CD34$^{+/-}$ group (n=7) are shown. SEM are indicated.

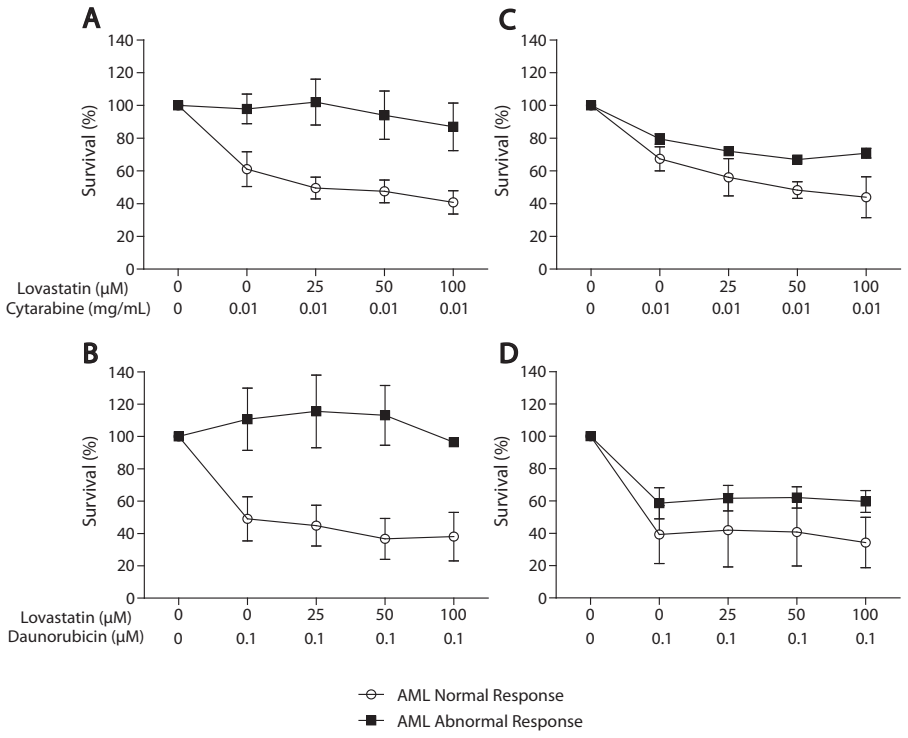

Figure 4. Effects of increasing concentrations of lovastatin in combination with chemotherapeutic drugs on in vitro survival of sorted CD34⁺CD38⁻ (A,B) and CD34⁺CD38⁺ (C,D) AML cells. Two groups are shown: normal responders (n=4) and abnormal responders (n=3). SEM are indicated.

cytarabine (data not shown). However, also here within the group of AMLs, different subgroups could be distinguished when looking at cell survival. Most AML CD34⁺ cells with a reduced statin sensitivity demonstrated also a diminished susceptibility for the combined use of statins plus cytarabine or daunorubicin (Figure 3). To investigate whether the observed effects were also noticed in the most primitive AML cell population, AML CD34⁺ cells were sorted into the CD34⁺CD38⁻ cell fraction and the CD34⁺CD38⁺ fraction (n=7). Four AML samples belonged to the normal responder group and three samples to the abnormal responder group. As depicted in Figure 4, the same distinction in responsiveness to cytostatic agents was observed in the absence or presence of lovastatin. Especially in the CD34⁺CD38⁻ subfraction the discrepancy between normal versus abnormal responders was most noticeable (e.g., for ARA-C in combination with 25 μM lovastatin, p=0.01 for CD34⁺CD38⁻ versus p=0.2 for CD34⁺CD38⁺; for DNR, p=0.05 versus p=0.5).

To verify whether similar results can be obtained in a clonogenic assay, normal and AML CD34⁺ cells were exposed to lovastatin and/or cytostatic agents for 24 hours and subsequently cultured in CFC medium. However, CD34⁺ cells from six out of seven AML samples did not show any *in vitro* colony formation in the CFC assay. Normal CD34⁺ cells (n=5) showed an inhibition of 12% and 8% with 25 μM lovastatin or daunorubicin, respectively, which increased to 36% inhibition when both agents were used (p=0.01, data not shown). Comparable results were obtained cytarabine (28% versus 60%, p=0.04).

Given the observed variability in effects of lovastatin on the CD34⁺ AML cells, we questioned whether this might be explained by a difference in expression or modulation of downstream targets. Therefore, the ABCG1 and ABCA1 mRNA expression levels were studied both in the normal responder and abnormal responder AML group and compared to normal CD34⁺ cells. A nonsignificant higher ABCG1 mRNA expression was observed in the normal responder AML group compared to the abnormal responder AML group and normal CD34⁺ cells. In addition, the lovastatin-induced ABCG1 mRNA expression was studied in the different groups. No statistically significant difference was found between normal CD34⁺ cells (0.4 fold downregulation, range 0.29-0.36) versus the normal and abnormal AML responder group, 0.2 (range minus 0.16-0.53), and 0.5 fold (range 0.16-0.70) downregulation respectively.

In vitro response pattern and clinical outcome

In view of the observed *in vitro* difference for statin sensitivity, we questioned whether these findings might correspond to patient characteristics or patient outcome. In the AML responder group, a normal or favorable cytogenetic pattern was found in 100% of the patients, whereas in the abnormal responder group, an unfavorable cytogenetic pattern was present in 50% of the patients. In the responder group, the median peripheral blast cell count at presentation was 16.6 × 10^9/L (range 2.2-128.3) and in the abnormal responder group 81.4 × 10^9/L (range 48.6-200, p=0.02). Based on these factors three prognostic risk groups can be defined, i.e., favorable risk (favorable cytogenetics in combination with a peripheral blood blast cell count <20 × 10^9/L at diagnosis), poor risk (those with poor-risk cytogenetics), and intermediate risk group (all others)[17,18]. In the responder group, 100% of the patients belonged to the good or intermediate risk group whereas in the abnormal responder group, this value was only 50% (Table 1). These findings correlated with treatment results. Complete remission on intensive chemotherapy was attained in 91% of the patients in the AML normal responder group with an EFS of 12.5 months (range 1-37). In the abnormal responders, CR was attained in 17% of the patients with a EFS of 0 months (range 0-15).

DISCUSSION

In the present study, we analyzed the *in vitro* effect of lovastatin on the survival of AML subpopulations in the absence or presence of conventional applied chemotherapeutics. Thus far, *in vitro* studies on statins in AML have been focused on the total AML cell population[6-9]. However, information of well-defined subpopulations might provide additional information especially regarding the primitive CD34$^+$ AML cells that are responsible for the ongoing *in vivo* propagation[18,19]. By studying two different subpopulations of AML cells (primitive CD34$^+$ cells and more mature CD34$^-$ cells), we were able to demonstrate a significant difference in lovastatin susceptibility. The inhibitory effects of lovastatin were especially observed on the primitive CD34$^+$ subpopulation. The effects on the more mature CD34$^-$ subpopulation were less pronounced, suggesting that the primitive cell fraction is more dependent on cholesterol synthesis, which is in line with the high expression of ABC transporters in these cells. In addition, there were increased although variable inhibitory effects by the combined use of lovastatin and cytotoxic agents in both the AML and normal CD34$^+$ cells.

Interestingly, we found that some AML CD34$^+$ cells followed a response pattern similar to normal CD34$^+$ cells whereas 35% of the AML CD34$^+$ cells showed no change. This could not be reversed in a number of cases by co-exposure the cells to chemotherapeutic agents. Interestingly, the distinction between normal and abnormal responders for the AML CD34$^+$ fraction was most pronounced in the CD34$^+$CD38$^-$ cell fraction, a population that is enriched for leukemic stem cells[20]. The difference in responsiveness between the normal and abnormal responder AML groups could not be explained by a difference in the percentage of AML CD34$^+$ cells. In addition, no difference in susceptibility for lovastatin-mediated upregulation of ABCG1 mRNA was observed which is a major downstream target of lovastatin. It is more conceivable that the difference is linked to additional cellular characteristics of the AML cells belonging to both groups. Patient characteristics demonstrated that the abnormal responder group had a significant higher peripheral blast cell count at presentation. Moreover the group was characterized by a high frequency of poor-risk cytogenetic abnormalities. The cause of the difference in susceptibility is not resolved but might be linked to differences in the farnesylation or geranylgeranylation pathways that are also affected by lovastatin[3,6]. It has been demonstrated that statins disrupt the localization and function of isoprenylated molecules in the cell membrane[3,6]. This change will result in an altered GTPase function of the Ras or Rho signaling that has an important impact on cellular processes such as proliferation and survival[21,22]. Alternatively, the difference may be linked to an altered expression of anti-apoptotic proteins between both AML groups. It has been described that overexpression of Bcl-xL protects against statin-induced

apoptosis in murine tubular cells[23]. Both of these possible explanations are currently under evaluation.

In summary, these results demonstrate distinct differences in lovastatin susceptibility between CD34⁺ and CD34⁻ in the normal and leukemic counterpart and between different AML subtypes. Additional studies will clarify whether the described *in vitro* assay can be used for *in vivo* identification of patients that might benefit from application of statins in combination with chemotherapeutic agents.

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4

Combining simvastatin with the farnesyltransferase inhibitor tipifarnib results in an enhanced cytotoxic effect in a subset of primary CD34$^+$ AML samples

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ABSTRACT

Purpose: To show whether the inhibitory effects of the cholesterol synthesis inhibitor simvastatin on human CD34$^+$ acute myeloid leukemia (AML) cells can be further promoted by combining it with the farnesyltransferase inhibitor tipifarnib. *Experimental design:* Normal CD34$^+$, AML CD34$^+$ and CD34$^-$ sorted subfractions, and AML cell lines (TF-1 and KG1a), were exposed to simvastatin and tipifarnib. *Results:* Both simvastatin and tipifarnib showed a cytotoxic effect on AML cell lines, which was additive when used in combination. In primary sorted CD34$^+$ AML cells a heterogeneous response pattern was observed upon treatment with simvastatin when analyzing cell survival. A group of normal (n=12) and abnormal (n=10) responders were identified within the AML CD34$^+$ subfraction when compared with normal CD34$^+$ cells. This distinction was not observed within the AML CD34$^-$ cell fraction. When the CD34$^+$ AML cells were exposed to simvastatin and tipifarnib, a significant enhanced inhibitory effect was shown exclusively in the normal AML responder group, whereas the AML CD34$^-$ cell fractions all showed an enhanced inhibitory effect. The observed heterogeneity in AML responsiveness could not be explained by differences in effects on cholesterol metabolism genes or extracellular signal-regulated kinase phosphorylation in response to simvastatin and tipifarnib treatment. *Conclusion:* The results suggest that combined treatment with statins and farnesyltransferase inhibitors may be beneficial for a subset of AML patients that can be defined by studying the AML CD34$^+$ fraction.

INTRODUCTION

Acute myeloid leukemia (AML) is a clonal hematopoietic disease, characterized by the accumulation of immature myeloid blasts in the bone marrow. A minor tumor subpopulation with self-renewal potential, referred to as leukemic stem cells is responsible for the sustained expansion of the leukemia[1,2]. Thus far, these leukemic stem cells are phenotypically characterized by $CD34^+CD38^-$ [3,4], but a recent study has challenged this view suggesting that leukemic stem cells might also belong to the $CD34^+CD38^+$ cell fraction[5].

Cholesterol synthesis and the processing of low-density lipoprotein (LDL) is hyperactive in AML[6,7], as indicated by high mRNA levels of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoAR) and LDL receptor (LDLR) as well as LDL uptake studies. These findings are in line with the high expression of key genes of cholesterol metabolism in $CD34^+CD38^-$ AML cells[8]. In addition, AML cells possess several mechanisms to protect them against the cytotoxic effects of chemotherapeutics, including a rapid increase in their cellular cholesterol levels after exposure to chemotherapeutic drugs[9,10]. Therefore, interfering with this protective mechanism potentially offers the opportunity to improve standard antileukemic treatment.

Statins, targeting HMG-CoAR, are widely used plasma cholesterol-lowering drugs. Statins inhibit cholesterol synthesis at the level of the conversion of mevalonate and, as a consequence, also inhibit the production of various byproducts of the mevalonate pathway. These byproducts include farnesyl and geranylgeranyl isoprenoids that are involved in the signaling of GTPases, including the small G protein Ras[9,11]. Ras GTPases must be transferred from the cytoplasm to the plasma membrane by isoprenylation to allow them to function as signal transducers[12-14]. Inhibiting farnesylation is of interest, because farnesylated proteins, particularly the protein products of the *RAS* gene family, are frequently activated in AML; e.g., by *RAS* mutations or due to the autocrine or paracrine production of growth factors[15]. However, the statin concentrations necessary to inhibit specific protein isoprenylation are 100-fold to 500-fold higher than those required to inhibit cholesterol synthesis[16].

The specific inhibition of Ras farnesylation can also be realized by the use of the Ras inhibitor tipifarnib. Tipifarnib is an oral nonpeptidomimetic Ras inhibitor, which selectively inhibits intracellular farnesyltransferase (FTase). Both statins as well as tipifarnib have been investigated as single agents in AML patients[17-21], resulting in modest response rates. Because alternative prenylation by geranylgeranyltransferase may bypass the inhibitory effect of tipifarnib[22] and statins are capable of blocking both geranylgeranylation and farnesylation, it is tempting to speculate that the combined use might have a more pronounced antileukemic effect. Therefore, sorted AML $CD34^+$ cells,

enriched for leukemic stem cells, were exposed to the cholesterol synthesis inhibitor simvastatin, the Ras inhibitor tipifarnib or to both compounds, and the findings were compared with normal CD34$^+$ cells. The results show that, given the heterogeneous response pattern between patient AML samples, a combination treatment with statins and farnesyltransferase inhibitors may be beneficial for around 50% of AML patients.

MATERIALS AND METHODS

Normal and AML hematopoietic cells and cell lines

Normal mobilized peripheral CD34$^+$ blood cells were collected from either healthy donors or patients awaiting autologous stem cell transplantation undergoing granulocyte colony-stimulating factor treatment, in accordance with institutional guidelines. After informed consent, bone marrow or peripheral blood cells were collected from AML patients at diagnosis. The Medical Ethical Committee of the University Medical Center Groningen (the Netherlands) approved the protocol. Patients were classified according to the French-American-British AML classification[23]. Mononuclear cells were enriched by density gradient centrifugation (Lymphocyte Separation Medium LSM 1077; PAA Laboratories GmbH, Cölbe, Germany) and freshly used or cryopreserved in RPMI 1640 (BioWhittaker, Brussels, Belgium) supplemented with 10% v/v fetal calf serum (FCS; Hyclone, Logan, UT) and 10% dimethyl sulfoxide (Merck, Amsterdam, the Netherlands), and stored at –196°C. Prior to analysis, the mononuclear cells were thawed, treated with DNase (Boehringer Mannheim, Almere, the Netherlands), washed, and incubated in RPMI 1640, supplemented with 10% FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin (ICN, Zoetermeer, the Netherlands), at 37°C and 5% CO_2. Cytogenetic analysis was done as described earlier[24]. The normal and AML samples were incubated with a fluorescein isothiocyanate (FITC)-conjugated antibody against CD34 and phycoerythrin (PE)-conjugated antibodies against CD14 and CD15 (Becton Dickinson, Alphen aan den Rijn, the Netherlands). Sorting of CD34$^+$ and CD34$^-$(CD14$^+$CD15$^+$) was done with the use of a MoFlo cell sorter (DakoCytomation, Carpinteria, CA).

The human AML cell line KG1a was cultured in Iscove's Modified Dulbecco's Medium (PAA Laboratories GmbH) supplemented with 10% FCS and 2 nM L-glutamine (ICN). The human erythroleukemic cell line TF-1 was cultured in RPMI 1640 supplemented with 10% FCS and 10 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF; Genetics Institute Inc, Cambridge, MA). Cultures were kept at 37°C and 5% CO_2.

Reagents

Simvastatin was obtained as a sodium salt from Merck Chemical Ltd. (Nottingham, UK) and dissolved in DMSO to obtain a 50 mM stock solution. R115777 (Tipifarnib, Zarnestra) was provided by Janssen Research Foundation and dissolved in DMSO to obtain a concentration of 10 mM.

Cell viability assay, annexin V and propidium iodide assay and cell-cycle analysis

Cell viability assays were done in duplicate according to the manufacturer's instructions with the use of the Cell Titer-Glo Luminescent cell viability assay (Promega, Madison, WI). Plates (96-well) were prepared with 100 µL RPMI 1640 medium supplemented with 10% FCS, and up to 10,000 cells per well were added (cell lines) or sorted ($CD34^+$ and $CD34^-$ AML cells). The cells were incubated with different concentrations of simvastatin (5, 25 and 50 µM) with and without different concentrations of tipifarnib (0.2- 5 µM) with the use of easy load pipet tips (Greiner Bio-One, Alphen aan den Rijn, the Netherlands) and analyzed after 24 (cell lines) or 48 (AML cells) hours.

Cell death was assessed with the use of an annexin V staining kit (IQ products, Groningen, the Netherlands) according to the manufacturer's recommendations. Briefly, after 48 hours of treatment with different concentrations of simvastatin and/or tipifarnib, cells were harvested, resuspended in 60 µL calcium buffer containing 3 µL of annexin V-FITC and incubated for 20 minutes at 4°C in the dark. Cells were washed with 2 mL calcium buffer and subsequently resuspended in 200 µL containing 1.7 µL propidium iodide (PI, Sigma). Binding of FITC-conjugated annexin V and PI was measured by fluorescence-activated cell sorting (FACS) analysis on a FACSCalibur (Becton Dickinson). Data were analyzed with the use of Winlist 3D (Verity Software House, Topsham, ME).

Cell-cycle analysis was done by determining the DNA content of cells by staining with PI (IQ products) in sodium citrate (1 mg/mL; Sigma-Aldrich, Zwijndrecht, the Netherlands) containing 100 µg/mL RNase A (Sigma-Aldrich), 20 µg/mL PI and 0.1% Triton X-100 (Sigma-Aldrich) for 60 minutes at room temperature. PI fluorescence was analyzed by FACS analysis. Cell cycle distributions were calculated with ModFit LT (Verity Software House).

Western blotting

TF-1 cells were treated in GM-SCF-free medium for 48 hours with simvastatin and/or tipifarnib, after which GM-CSF (10 ng/mL) was added for 15 minutes. Unsorted mononuclear AML cell fractions were cultured in RPMI 1640 supplemented with 10% FCS and treated for 24 hours. Whole cell extracts were obtained by lysing 5×10^5 cells in boiling Laemmli sample buffer for 5 minutes. Samples were separated by 10% SDS-PAGE

and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) in Tris-buffer with the use of a semidry electroblotter from Bio-Rad Laboratories (Veenendaal, the Netherlands). Membranes were probed with antibodies according to the manufacturer's protocols. The antibodies used were extracellular signal-regulated kinase (ERK) 1/2 (K23; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and phospho-ERK1/2 (Thr202/Tyr204; Cell Signaling Technology Inc., Beverly, MA). Immunodetection of phospho-ERK and total ERK was done according to standard procedures, and binding of antibodies was detected by enhanced chemiluminescence. Densitometry was carried out with the use of ImageJ.

Quantitative real-time PCR

Total RNA was isolated with the use of the RNeasy mini kit (Qiagen, Venlo, the Netherlands) and was reverse transcribed with the use of RevertAid™ H Minus M-MuLV reverse transcriptase (Fermentas, St Leon-Roth, Germany). Quantitative PCR was done with the use of the ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Primers and probes for the human ATP-binding cassette (ABC) transporters and cholesterol metabolism genes were used as described before[8,25]. As endogenous control, the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used. The primers were obtained from Invitrogen (Breda, the Netherlands). The probes were labeled by a 5' 6-carboxyfluorescein (FAM) reporter and quenched by 6-carboxytetramethylrhodamine (TAMRA) at its 3' end (Eurogentec, Maastricht, the Netherlands). We used 4 µL of diluted cDNA in each PCR reaction in a final volume of 20 µL, containing 900 nM of sense and antisense primers, 200 nM of the Taqman probe, 5 mM $MgCl_2$, KCl, TrisHCl, 0.2 mM dATP, dCTP, dGTP, dTTP, and dUTP, and 0.5 U of AmpliTaq DNA polymerase (qPCR Core Kit, Eurogentec). The PCR program was 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The expression of the genes was standardized for the expression of GAPDH. Serial cDNA dilutions of the AML samples were used to generate calibration curves. The expression of each gene in each sample was analyzed in duplicate.

Statistical analysis

Student's *t*-test was used to calculate the differences between cell line samples; Friedman's and Mann-Whitney's nonparametric tests were done to calculate significant differences between AML samples. Data were expressed as mean ± SD or SEM, as indicated. All *p* values are given for two-sided tests and $p \leq 0.05$ was considered significant. Additivity was defined as an increased effect of the combination exposure compared with the single exposure of simvastatin or tipifarnib. Normal $CD34^+$ AML responders were defined as follows: at the concentration of either simvastatin or

tipifarnib at which the first significant effect was observed (i.e., 25 μM simvastatin and 1 μM tipifarnib) with the use of the cell viability assay, the decrease in viability of the normal CD34⁺ cells ± standard deviation was considered as normal. AML CD34⁺ cells with viabilities above this value were considered to give an abnormal response.

RESULTS

Patient characteristics

The 22 AML patients studied, with a median age of 52 years (range 19-79), were classified as the French-American-British classification groups M0 (n=1), M1 (n=6), M2 (n=5), and M4/5 (n=10). The clinical characteristics of the patients are shown in Table 1.

Table 1. Clinical and cellular characteristics of AML patients

AMLs	FAB Class.	Leukocytes at presentation (×10⁹/L)	CD34⁺* (%)	Cytogenetics†	Normal responder to simva in CD34⁺ cells‡	Normal responder to simva + tipi in CD34⁺ cells‡
1	M2	56	29	N	Yes	Yes
2	M1	200	87	inv(3q),7-,10-	Yes	Yes
3	M5	109	1	ND	Yes	Yes
4	M5b	25	28	N	Yes	Yes
5	M5b	97	15	N	Yes	Yes
6	M4	89	43	inv(16)	Yes	Yes
7	M1	128	24	N	Yes	Yes
8	M2	7	28	N	Yes	Yes
9	M1	17	40	N	Yes	Yes
10	M1	36	80	N	Yes	No
11	M5	102	60	inv(16)	Yes	No
12	M2	2	36	N	Yes	No
13	M4/M5	50	7	N	No	Yes
14	M5	67	8	3q-, 5q+,+8	No	Yes
15	M5b	220	23	t(11;20)	No	No
16	M2	59	17	del9 q12q22	No	No
17	M2	10	23	N	No	No
18	M1	64	35	t(6;9), trisomy 13	No	No
19	M1	96	92	N	No	No
20	M5a	49	85	N	No	No
21	M0	102	90	5q-, trisomy 6	No	No
22	M5a	8	11	46N,xy +11q23	No	No

* Percentage of CD34⁺ cells in the AML mononuclear cell fraction.

† Cytogenetics: N: normal; inv: inversion; ND: not determined.

‡ Normal responders (Yes) and abnormal responders (No) to simvastatin and tipifarnib were defined as described in materials and methods.

The median peripheral blast cell count at diagnosis was 62 × 10^9/L (range 2-220). The majority of patients were treated according to ongoing HOVON protocols, i.e., for patients <60 years the HOVON SAKK AML-42 study[26] and for patients >60 years the HOVON 43 study[27]. Palliative treatment consisting of treatment with 6-mercaptopurine[28] was given to patients who were ineligible for intensive chemotherapy (n=3).

Tipifarnib and simvastatin decrease cell viability by inducing apoptosis and cell cycle arrest

First, the effects of tipifarnib and simvastatin on the hematopoietic cell lines KG1a and TF-1 were assessed. With simvastatin alone, a dose-dependent decrease in cell survival of up to 30% was shown (Figure 1) in both cell lines. Similar results were obtained with tipifarnib (up to 60% decrease of survival), whereas the combined use showed an even more efficient decrease of up to 75% in cell viability, which was additive compared with either treatment alone. To define whether this decline in cell survival was due to cell cycle arrest or apoptosis, cell cycle status and cell survival were defined by PI staining and an annexin V/PI assay, respectively. Treatment of TF1 cells with tipifarnib increased the number of cells in the G2/M cell cycle phase (9% versus 27% at 0.2 μM; p=0.002), and lowered the number of cells in the G0/G1 phase (51% versus 36% at 0.2 μM; p=0.049; Figure 2A). Cells in S phase remained the same. Treatment with 50 μM simvastatin resulted in fewer cells in the S phase (40% versus 24%) and more in G0/G1 (51% versus 69%; Figure 2A), but these differences did not reach significance (p=0.2). When combining both treatments, we observed an inhibitory effect on the cells in the S phase (p=0.02), and there were more cells in G0/G1 phase (p=0.09) or G2/M phase (p=0.05) when using tipifarnib and simvastatin at a dose of 5 μM and 50 μM (Figure 2A).

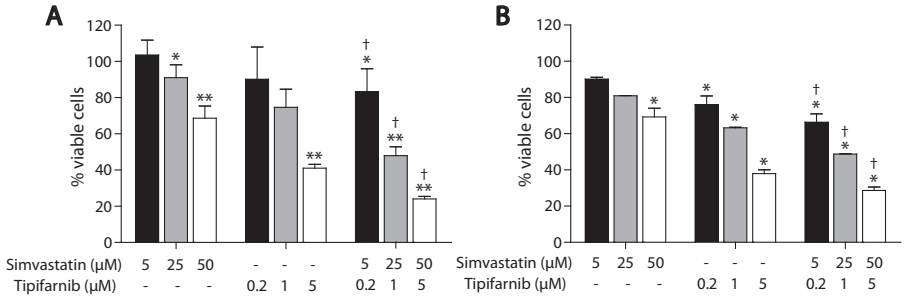

Figure 1. Cell viability of TF-1 (A; n=4) and KG1a (B; n=2) treated for 48 hours with tipifarnib, simvastatin, or the combination. Data are shown as the percentage of ATP levels compared with control cells. Data shown are mean values ± SD, *p<0.05, **p<0.001; †addition.

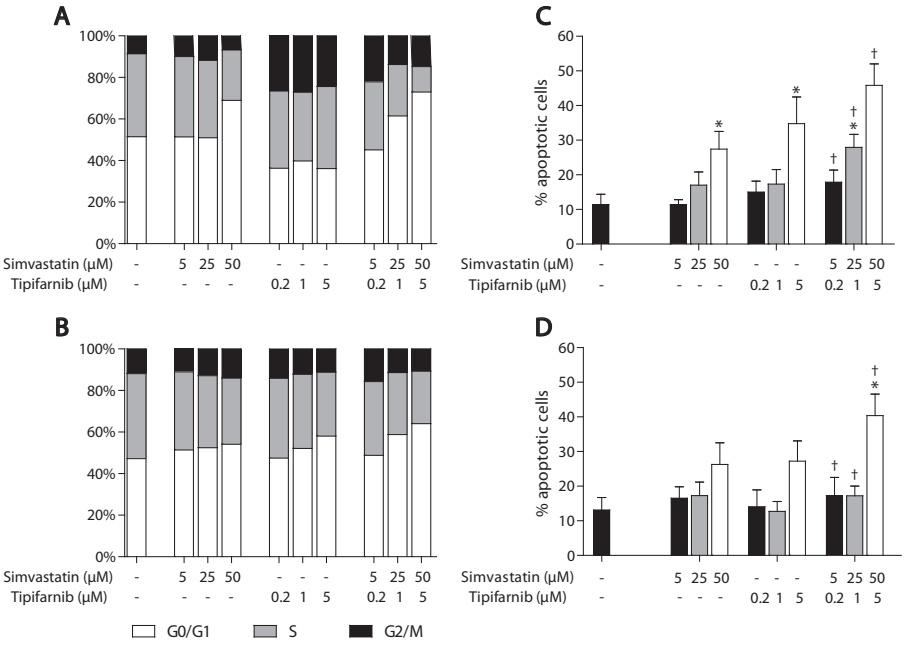

Figure 2. The effect of simvastatin and tipifarnib on cell cycle status and cell death in TF-1 and KG1a cells after 48 hours of treatment. Percentage of TF-1 (A) and KG1a (B) cells in Go/G1, S and G2/M phases are shown after treatment with either tipifarnib or simvastatin alone, or after combination treatment. Data are shown as a mean of 3 independent experiments. The effect of simvastatin and tipifarnib treatment cell death in TF-1 (C) and KG1a (D) cells after 48 hours of treatment. Data are represented as a percentage of (late) apoptotic cells. The average results of five and four independent experiments are shown for TF-1 and KG1a, respectively. *p<0.05; †addition.

In KG1a cells, we observed similar effects: combination of both treatments resulted in an increase of cells in G0/G1 phase (Figure 2B; 47% versus 64% at 50 μM simvastatin and 5 μM tipifarnib; p=0.002) and a decrease of cells in S phase (41% versus 25%; p<0.001). Thus, treatment of TF-1 and KG1a cells with simvastatin and tipifarnib results in a G0/G1 cell cycle arrest. It seemed that this cell cycle arrest was associated with an increased number of cells in apoptosis, which was especially noticed for the TF-1 cell line (Figure 2C,D). Also here we found additive effects when combining both treatments.

The effect of simvastatin and tipifarnib on primitive CD34⁺ normal and AML cells

To show whether in patient AML cells comparable effects can be noticed, AML mononuclear cells (n=22) were sorted into CD34⁺ and CD34⁻ subfractions and exposed to the two agents. The results were compared with the effects seen in normal CD34⁺ (n=8). When exposed to simvastatin (Figure 3A) or tipifarnib (data not shown), normal CD34⁺

cells showed a concentration-dependent decrease in cell survival, which was significant at a concentration of 25 μM simvastatin (p<0.001) and 1 μM tipifarnib (p=0.01). Treatment of normal CD34$^+$ cells with 25 μM simvastatin in combination with tipifarnib showed an additive inhibitory effect on cell survival compared with the effects of the separate compounds (Figure 3B). However, within the CD34$^+$ AML cell fraction, a marked variability in responsiveness could be observed. Based on the response pattern of normal CD34$^+$ cells, two AML subgroups could be distinguished (see materials and methods) when the CD34$^+$ AML cells were exposed to simvastatin. Fifty-five percent (n=12) of the AML CD34$^+$ cells had a response pattern comparable to normal CD34$^+$ (e.g., p=0.6 at 25 μM) whereas 45% (n=10) of the AML CD34$^+$ cells showed reduced simvastatin sensitivity (Figure 3A; e.g., p=0.003 at 25 μM). In contrast, the CD34$^-$ AML subfraction showed a response pattern comparable to normal CD34$^+$ cells, and no difference was observed between normal and abnormal responders within CD34$^-$ AML cells (e.g., p=0.6 at 25 μM).

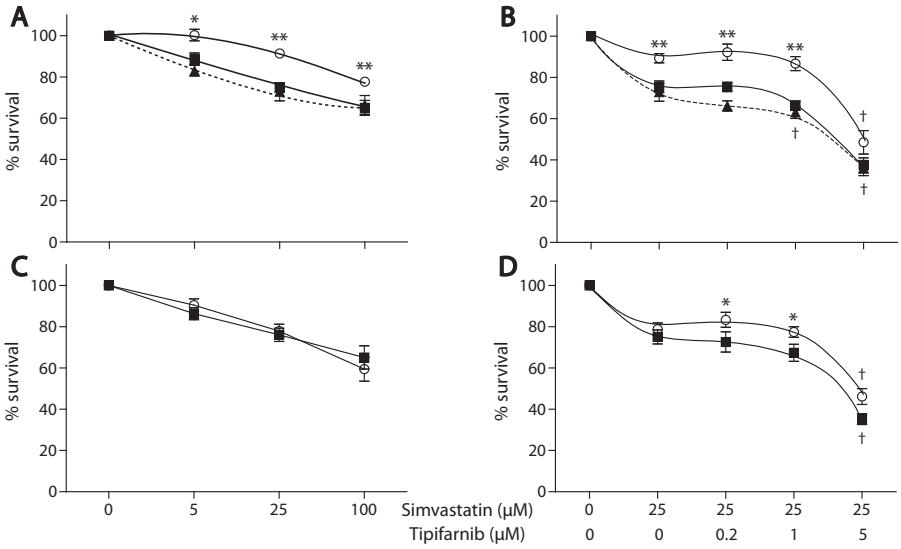

Figure 3. Effect of simvastatin and tipifarnib on in vitro survival of normal and AML CD34$^+$ (A, B) and CD34$^-$ (C, D) AML cells. Two groups of AML patients, consisting of either normal or abnormal responders, and a control group are shown. The responder group is defined as having a decrease in cell viability which is mean ± SD of control cells at 25 μM simvastatin or 25 μM simvastatin + 1 μM tipifarnib. Normal CD34$^+$ cells (PBSCs) n=8; AML normal responders n=12 (A) or n=11 (B); AML abnormal responders n=10 (A) or n=11 (B). SEM is indicated. *p<0.05 and **p<0.01 normal versus abnormal responder; $^\dagger p$<0.01 versus 25 μM simvastatin alone.

Next, we studied whether the suppressive effects of simvastatin on AML cells can further be promoted by cotreatment with tipifarnib as shown for normal CD34⁺ cells. The results show that 50% (n=11) of the AML CD34⁺ cells had a response pattern comparable to normal CD34⁺ cells, whereas the other half of the AML CD34⁺ cells showed reduced sensitivity for the combined treatment of simvastatin (25 μM) and tipifarnib (1 μM; Figure 3B; $p<0.001$). It appeared that 8 of 10 of the AMLs that were not responsive to simvastatin were also not affected when both compounds were used. When the abnormal responder group was exposed to 1 μM tipifarnib and 25 μM simvastatin, the decrease in cell survival was not enhanced compared with the effect of simvastatin alone ($p=0.5$). In contrast, in the AML CD34⁺ responder group, a significant enhancement in cell death was observed when both compounds were used at low dose (1 μM; $p=0.01$). At higher concentrations, a strong reduction in cell survival was observed in normal CD34⁺ cells, as well as in the AML abnormal and normal responder group, but these concentrations are physiologically not relevant. The AML CD34⁻ subpopulation showed a comparable response pattern to normal CD34⁺ cells, and no clear distinction could be made between responders and abnormal responders when both compounds were used ($p=0.03$ and $p=0.05$ for 0.2 μM and 1 μM tipifarnib, respectively; Figure 3D).

Heterogeneity in AML response pattern is not related to differences in modulation of cholesterol metabolism genes and ERK phosphorylation by simvastatin and tipifarnib

To investigate whether the functionality of either simvastatin or tipifarnib was different in the normal versus the abnormal responder group, we studied important downstream targets of simvastatin and tipifarnib. The functionality of simvastatin was tested based on reported upregulation of HMG-CoAR and LDLR and a downregulation of ABCA1 and ABCG1 at the mRNA level[29,30], whereas the downstream effects of tipifarnib were analyzed by studying the change in ERK1/2 phosphorylation, a downstream target of Ras. In the 6 AML samples (3 normal responders and 3 abnormal responders to simvastatin, tipifarnib, and both drugs) studied, the expression of HMG-CoAR and LDLR was almost 2.5-fold higher after treatment with 25 μM simvastatin (Figure 4A; $p=0.001$ and $p=0.003$, respectively), and ABCA1 and ABCG1 expression was 2-fold decreased ($p<0.001$). Treatment with tipifarnib did not alter mRNA expression, and the combination of tipifarnib and simvastatin had no additive effect compared with treatment with simvastatin alone (data not shown).

Tipifarnib (0.5 and 1 μM) did not affect ERK phosphorylation in any of the studied AMLs of the normal responder or abnormal responder group (Figure 4B). Also simvastatin did not lead to inhibition of ERK-phosphorylation. However, combining simvastatin and tipifarnib resulted, in 69% of the 16 tested AML samples, in a decrease of phospho-ERK expression (88% ± 10% for 1 μM tipifarnib and 25 μM simvastatin; n=11)

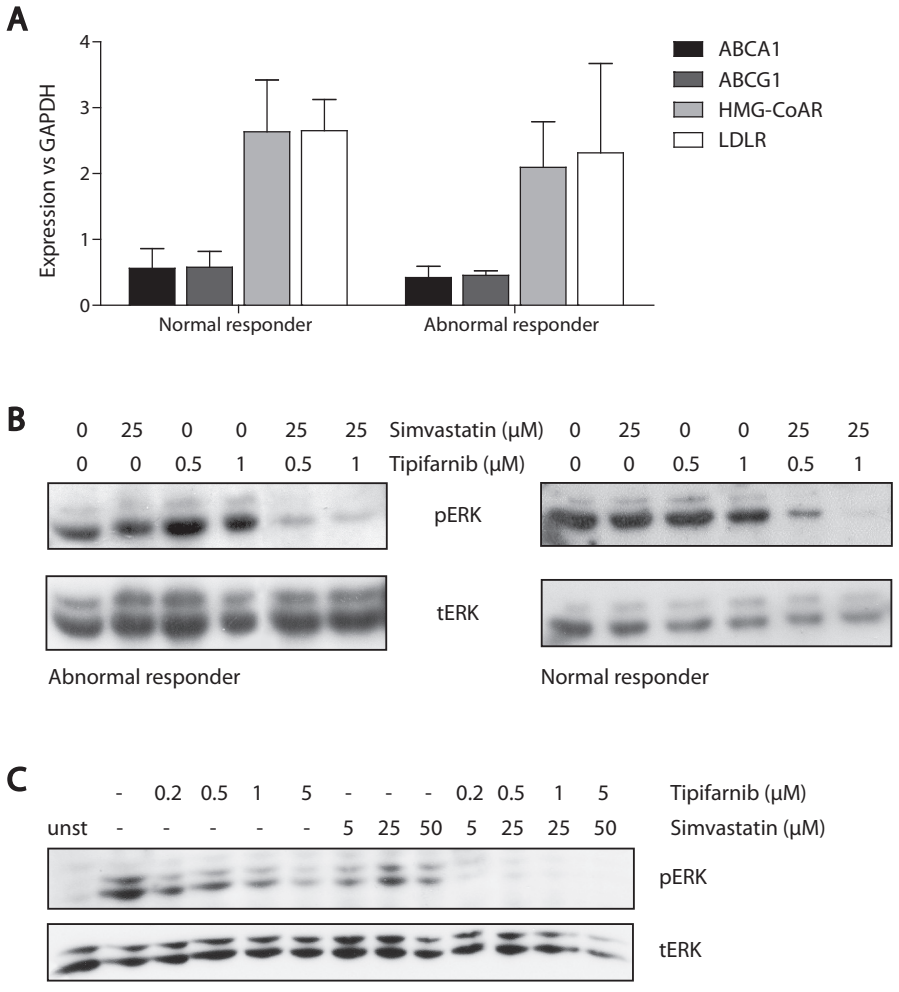

Figure 4. Effect of simvastatin and tipifarnib on mRNA expression levels and ERK phosphorylation in primary AML samples and TF-1 cells. (A) Cells were treated for 24 hours with 25 μM simvastatin. mRNA expression levels of cholesterol metabolism genes in total mononuclear cell (MNC) fractions of normal (n=3) and abnormal (n=3) responder AML samples are shown. Results are displayed as mean ± SD, compared with untreated cells, which were set to 1. Data were normalized to GAPDH. (B) Effects of tipifarnib and simvastatin on phosphorylation of ERK in AML samples. The total MNC fraction was incubated with 0.5 and 1 μM tipifarnib and/or 25 μM simvastatin for 48 hours before harvesting. Anti-ERK was used as a loading control. This experiment is representative of 7 abnormal responder and 4 normal responder AMLs. (C). Effects of tipifarnib and simvastatin on phosphorylation of ERK in TF-1 cells. Cells were deprived of GM-CSF, incubated with tipifarnib and simvastatin for 48 hours, and stimulated with GM-CSF for 15 minutes. 'Unst' indicates unstimulated cells. Anti-ERK was used as a loading control.

irrespective of the AML used. Similar results were observed when TF-1 cells were cultured with GM-CSF and incubated with simvastatin and tipifarnib (Figure 4C).

In vitro response pattern and patient characteristics

In view of the observed *in vitro* difference for simvastatin and tipifarnib sensitivity we questioned whether these findings might correspond to patient characteristics. In the group responding *in vitro* to both tipifarnib and simvastatin, the median percentage of CD34⁺ cells in the AML mononuclear cell fraction at presentation was 28% (mean 28; range 1-87), and in the non-responder group 50% (mean 36; range 11-92; p=0.08; Table 1). No correlation was found between AML responder groups and cytogenetics and peripheral blast count. The French-American-British classifications were distributed equally over the normal and abnormal responder groups.

DISCUSSION

In this study, we addressed the question of whether the combined use of a cholesterol synthesis inhibitor and a farnesyltransferase inhibitor might have an augmented cytotoxic effect on CD34⁺ AML cells. The results show that, in human CD34⁺ AML leukemic cell lines, simvastatin and tipifarnib have an enhanced suppressive effect on cell survival compared with either compound alone, which was characterized by an inhibition of cell cycle progression and enhanced apoptosis. Several studies have shown that geranylgeranylated proteins, not farnesylated proteins, are required for the G1 to S phase transition[31] and that statins are able to block cell cycle in G1 due to geranylgeranylation rather than farnesylation[32]. Apparently, this mechanism varies among different cell types. We observed G1 cell cycle arrest after treatment with both simvastatin and tipifarnib, whereas only a limited effect was observed with either compound alone. This suggests that farnesylated proteins are also responsible for cell cycle progression in AML cells. Likewise, ERK phosphorylation was strongly inhibited only when both simvastatin and tipifarnib were used. It may be that geranylgeranylation of Ras can overcome the inhibitory effects of tipifarnib on Ras farnesylation, and both geranylgeranylation and farnesylation must be blocked to decrease Ras activation. This is consistent with the idea that K-Ras and N-Ras can be both farnesylated and geranylgeranylated[22].

In contrast with AML cell lines, the observed inhibitory effect of simvastatin and tipifarnib was not found to be a general phenomenon for all primary AML samples. Most studies on statins have been focused on the total AML mononuclear cell fraction[33-35]. In the total mononuclear AML fraction a heterogeneous response to lovastatin has been

observed[33]. Our data show that a heterogeneous response also exists within the more homogeneous primitive AML CD34$^+$ subfraction. Two subgroups could be defined based on sensitivity to simvastatin alone. Almost 50% of the AML CD34$^+$ showed a reduced sensitivity to simvastatin, tipifarnib, or to both compared with normal CD34$^+$ cells, whereas the remaining AML samples showed a response pattern comparable to normal CD34$^+$ cells.

A remarkable finding was the fact that, within the AML CD34$^-$ subpopulation, no distinction could be made between normal and abnormal responders. These findings show that immature AML cells are intrinsically different from the CD34$^-$ cell population. This is in line with previous studies showing that the total AML cell fraction is a heterogeneous cell population[3,36], that leukemic stem cells belong to the CD34$^+$ cell population[1,37], and that CD34$^+$ AML cells have almost exclusive self-renewal properties as defined in long-term cell culture assays as well as engraftment studies in severe combined immunodeficient mice[38,39]. Moreover, it shows that by using the total mononuclear cell population, the differences in response would have been unnoticed, especially when the total AML cell fraction comprises a low percentage of CD34$^+$ cells.

Several clinical studies have already been done with either statins or with tipifarnib[17,19-21,40]. In AML, statins have been combined with intensive chemotherapy whereas tipifarnib has been used as single agent. Phase 1 and 2 studies have shown that only a minority of the patients were responsive to treatment. Likewise, the results of the present study suggest that, *in vitro,* not all AML patients are responsive to clinically relevant concentrations of simvastatin and tipifarnib[17,19], and that this distinction can be made by using exclusively the CD34$^+$ fraction. Therefore, it might be useful to determine whether the *in vitro* response is predictable for clinical response in order to be able to select patients who are eligible for treatment.

Importantly, to be able to predict response, the rationale behind the difference in susceptibility of AML cells to simvastatin and tipifarnib should be elucidated. We observed a considerable overlap between the simvastatin and tipifarnib responders, and no differences on studied downstream targets of simvastatin and tipifarnib were observed, suggesting that there might be a common mechanism of resistance.

It is conceivable that a specific set of anti-apoptotic proteins are responsible for the differences in response. It has been shown that sorted CD34$^+$ AML cells are more resistant to (spontaneous) apoptosis than the corresponding CD34$^-$ fractions, which is paralleled by higher Bcl-2, Bcl-xL, Mcl-1, P-gp, and low Bax expression levels[41]. In addition, it has been shown that overexpression of Bcl-xL and Bcl-2 protects against statin-induced apoptosis[42,43]. It is unlikely that simvastatin and tipifarnib responsiveness correlates with the Ras activity status of the AML cells, because both normal and abnormal responders show a basal phosphorylation level of ERK and a decrease of

ERK activity upon combination treatment, which is in line with the results of Stirewalt et al.[11], who suggested that neither Ras mutations, nor high Ras protein expression are found to be consistently associated with increased statin sensitivity. Likewise, in clinical studies using tipifarnib in AML, the Ras mutational status and inhibition of phophorylated ERK did not correlate with clinical responsiveness to tipifarnib[19,20].

Thus far, the clinical data on the use of either agent are encouraging, but not convincing. Because we observed *in vitro* significant cytotoxic effects in an AML subgroup with clinically achievable concentrations, we predict that combining both agents *in vivo* will be advantageous in a subset of AML patients.

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5

Heterogeneity in simvastatin-induced cytotoxicity in AML is caused by differences in Ras-isoprenylation

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A short version of this chapter is accepted for publication in Leukemia

ABSTRACT

3-Hydroxy-3-methylglutaryl CoA reductase is the rate-controlling enzyme of the mevalonate pathway that yields cholesterol as well as isoprenoids, such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). Inhibition of HMG-CoAR activity by statins revealed differences in cytotoxic effects among patients with acute myeloid leukemia (AML). We studied the mechanism underlying this heterogeneity by comparing AML cells with different sensitivities to simvastatin and showed that heterogeneity in simvastatin-induced cytotoxicity was not due to differential effects on cholesterol synthesis. Yet, simvastatin-induced cytotoxicity in sensitive cell lines (half maximal effective concentration (EC_{50}) 1-5 μM), and in a subset of patient AML samples (n=11) could effectively (up to 100%) be prevented by cotreatment with FPP and GGPP, whereas insensitive cell lines (EC_{50} 50-100 μM) were not notably protected (maximal 33%). Similarly, inhibition of isoprenylation in AML cell lines by either simvastatin or specific inhibitors of the isoprenylation pathway demonstrated that the heterogeneity in response to simvastatin was related to the extent of isoprenylation inhibition and to sensitivity to ERK inhibition. These data indicate that interference with the Ras-isoprenylation route, rather than with the cholesterol synthesis route, determines simvastatin-induced cytotoxicity in AML, which may provide means to predict its clinical usefulness in AML patients.

INTRODUCTION

Acute myeloid leukemia (AML) is characterized by the accumulation of immature myeloid blasts in the bone marrow. Different mechanisms have been identified that protect AML cells against cytotoxic actions of chemotherapy, including dysregulation of cellular cholesterol homeostasis[1,2]. Both synthesis and influx of cholesterol are increased in AML cells, as indicated by increased 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoAR) and low-density lipoprotein receptor (LDLR) mRNA expression and protein activity in these cells[3-5]. In addition, AML cells increase their cholesterol contents upon *in vitro* exposure to chemotherapeutic drugs, which might render them less susceptible to the effects of these cytotoxic agents[1,6]. Therefore, interfering with cholesterol metabolism, by using statins to suppress cholesterol synthesis, has been proposed as an opportunity to improve antileukemic treatment.

Statins (e.g., simvastatin) are widely used plasma cholesterol-lowering drugs, that inhibit cholesterol synthesis at the level of HMG-CoAR by blocking the conversion of HMG-CoA to mevalonate (Figure 1). Mevalonate is also precursor of isoprenoids such as farnesyl and geranylgeranyl. Farnesylation or geranylgeranylation, collectively referred to as isoprenylation, is required for the attachment of small GTPases (e.g., Rho, Ras) to the plasma membrane and their subsequent participation in signal transduction pathways that regulate growth and survival. Examples hereof are the PI3K/Akt and Ras/MEK/ERK pathways[1,7,8]. Isoprenylation is catalyzed by geranylgeranyltransferase (GGTase) or farnesyltransferase (FTase)[9,10]. Whereas Rho only undergoes geranylgeranylation, H-Ras can be farnesylated and N-Ras and K-Ras can be both farnesylated and geranylgeranylated[11,12]. Particularly the protein products of the *RAS* gene family are frequently activated in AML cells by mutations or due to autocrine or paracrine production of growth factors[13].

Statins induce cell death in various human AML cell lines as well as in primary human AML cells *in vitro*[14-18]. Different mechanisms can be responsible for simvastatin-mediated cell death. Simvastatin may exert its cytotoxic effects by blocking cholesterol synthesis, or by inhibiting the actions of signaling molecules, such as Ras, by preventing their isoprenylation[1,7,19,20]. However, the exact mechanism of simvastatin-induced cytotoxicity has remained elusive thus far.

We recently observed a heterogeneity in cytotoxicity among primary human AML samples upon *in vitro* treatment with simvastatin[15], suggesting that only a subset of AML patients may benefit from statin treatment. In order to be able to predict which patients may benefit from statin treatment, we investigated whether differences in simvastatin-induced cytotoxicity are confined to differential effects on the cholesterol synthesis route, or on the isoprenylation route. We show in human AML cell lines and

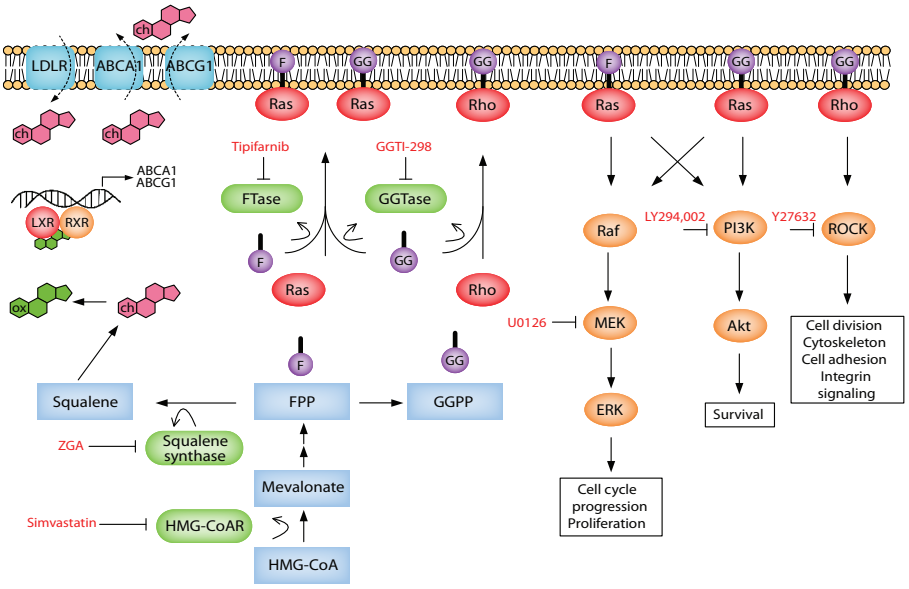


Figure 1. The mevalonate pathway. The mevalonate pathway, that can be inhibited by simvastatin, leads to the synthesis of cholesterol and the isoprenoids FPP and GGPP. Oxidized cholesterol (oxysterol) binds to the nuclear receptor LXR, which leads to the transcription of ABC transporters ABCA1 and ABCG1. These transporters are together responsible for cholesterol efflux. LDLR mediates cholesterol influx. Isoprenylation with FPP or GGPP is required for the plasma membrane binding of small GTPases such as Rho and Ras, in order to activate pathways involved in proliferation and survival. Ch: cholesterol; F: farnesyl pyrophosphate; FTase: farnesyltransferase; GG: geranylgeranyl pyrophosphate; GGTase: geranylgeranyl transferase; LDLR: low-density lipoprotein (LDL) receptor; LXR: liver X receptor; Ox: oxysterol; RXR: retinoid X receptor; ZGA: zaragozic acid A.

primary AML cells that inhibition of the isoprenylation route, rather than the cholesterol synthesis route, by simvastatin is responsible for its cytotoxic effects. Heterogeneity in simvastatin-induced cytotoxicity in AML cell lines was shown to be linked to the extent of inhibition of isoprenylation, as well as to sensitivity to isoprenylation inhibitors, and more specifically to the degree of phospho-ERK inhibition.

MATERIALS AND METHODS

Human AML hematopoietic cell lines and primary AML samples

The human myeloid leukemia cell lines HL60 and NB4 were cultured in RPMI 1640 (Lonza, Breda, the Netherlands) supplemented with 10% fetal calf serum (FCS; PAA

Laboratories GmbH, Cölbe, Germany). TF-1 was cultured in RPMI 1640 supplemented with 10% FCS and 10 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF; Genetics Institute Inc, Cambridge, MA), KG1a in Iscove's Modified Dulbecco's Medium (IMDM; PAA Laboratories GmbH) supplemented with 10% FCS and 2 nM L-glutamine (ICN, Zoetermeer, the Netherlands), and UT7-GM in IMDM with 10% FCS and 10 ng/mL GM-CSF. OCI-M3 (OCI/AML3) was cultured in α-MEM (Lonza) supplemented with 10% FCS. Cultures were kept at 37°C and 5% CO_2. Bone marrow or peripheral blood cells of AML patients were collected and cryopreserved as described earlier[15]. The Medical Ethical Committee of the University Medical Center Groningen approved the protocol. Patients were classified according to the French-American-British AML classification[21]. Cytogenetic analysis was done as described earlier[22]. Prior to analysis, mononuclear cells were thawed, treated with DNase (Boehringer Mannheim, Almere, the Netherlands), washed, and incubated in α-MEM, supplemented with 12.5% FCS, 12.5% horse serum, 20 ng/mL IL-3, 20 ng/mL granulocyte colony-stimulating factor, and 20 ng/mL thrombopoietin at 37°C and 5% CO_2.

Reagents

Simvastatin and GGTI-298 were obtained from Merck Chemical Ltd (Nottingham, UK). Tipifarnib was provided by Janssen Research Foundation. Zaragozic acid A, squalene, mevalonate, farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP) were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands). ROCK inhibitor Y27632 was purchased from Cayman Chemical, MEK inhibitor U0126 was obtained from Promega (Madison, WI) and PI3K inhibitor LY294,002 from Enzo Life Sciences (Raamsdonksveer, the Netherlands).

Cytotoxicity assay

Cytotoxicity was determined with the use of the Cell Titer-Glo Luminescent cell viability assay (Promega) according to the manufacturer's instructions. Plates (96-well) were prepared with 10,000 cells per well in 100 μL medium supplemented as described above. Upon treatment with simvastatin, the half maximal effective concentration (EC_{50}) in the various cell lines was determined as the concentration at which cytotoxicity was increased with 50% compared with untreated cells. To determine which agents could prevent simvastatin-induced cytotoxicity, the cells were incubated with simvastatin alone (for concentrations see figure legends), or in the presence of mevalonate, FPP, GGPP, or squalene. Fixed concentrations of mevalonate (250 μM), FPP (20 μM) and GGPP (10 μM) were used. These concentrations were optimal for preventing simvastatin-induced cytotoxicity (data not shown). To mimic the simvastatin-induced

cytotoxicity, cells were incubated with zaragozic acid A, GGTI-298, tipifarnib, U0126, Y27632, or LY294,002. After 48 hours, viability was assessed in duplicate.

Quantitative real-time PCR

Total RNA was isolated with the use of the RNeasy mini kit (Qiagen, Venlo, the Netherlands) and reverse transcribed with the use of RevertAid™ H Minus M-MuLV reverse transcriptase (Fermentas, St Leon-Roth, Germany). Quantitative PCR was done with the use of the ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Primers and probes for the human ABC transporters and HMG-CoAR and LDLR were described earlier[23]. As endogenous control the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used. The primers were obtained from Invitrogen (Breda, the Netherlands). The probes were labeled by a 5' 6-carboxyfluorescein (FAM) reporter and quenched by 6-carboxytetramethylrhodamine (TAMRA) at its 3' end (Eurogentec, Maastricht, the Netherlands). We used 3 µL of diluted cDNA in each PCR reaction in a final volume of 15 µL, containing 900 nM of sense and antisense primers, 200 nM of the Taqman probe, 5 mM $MgCl_2$, KCl, TrisHCl, 0.2 mM dATP, dCTP, dGTP, dTTP, and dUTP, and 0.5 U of AmpliTaq DNA polymerase (qPCR Core Kit, Eurogentec). The PCR program was 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The expression of genes was standardized for the expression of GAPDH. Serial cDNA dilutions of the AML samples were used to generate calibration curves. The expression of each gene in each sample was analyzed in duplicate.

Western blotting

For phospho-ERK expression determination, cells (0.5×10^6 per mL) were cultured in medium without GM-CSF, supplemented with 5% FCS in the presence of simvastatin and mevalonate, GGPP, or FPP. After 48 hours, GM-CSF (10 ng/mL) was added for 15 minutes. Whole cell extracts were obtained by lysing 5×10^5 cells in boiling Laemmli sample buffer for 5 minutes. Proteins were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) in Tris-buffer, with the use of a semidry electroblotter from Bio-Rad Laboratories (Veenendaal, the Netherlands). After blocking in 0.1% Tween-20 and 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS), membranes were probed with antibodies according to the manufacturer's protocols. Antibodies used were ERK1/2 (K23,;Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and phospho-ERK1/2 (Thr202/Tyr204; Cell Signaling Technology Inc., Beverly, MA). Antibody binding was visualized with enhanced chemiluminescence detection after incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody (Dako, Glostrup, Denmark).

For DnaJ, Rap1, and Ras expression, cells (0.5×10^6 per mL) were cultured as described above, in the presence of simvastatin, GGTI-298, or tipifarnib. Proteins were separated by 10% SDS-PAGE, and transferred to a PVDF membrane as described above. After blocking in 0.1% Tween-20, 5% skim powdered milk, and 2% BSA in TBS, membranes were probed with antibodies against DnaJ (HDJ-2; Labvision, Fremont, CA), Rap1a, N-Ras or β-actin (Santa Cruz Biotechnology, Inc.). The unprocessed form of DnaJ displays reduced mobility in SDS-PAGE compared with its processed counterpart. Unprocessed Rap1 was determined with the use of an antibody that exclusively detects the unprocessed form (Rap1a). Antibody binding was visualized with enhanced chemiluminescence detection, or with the use of an Odyssey infrared scanner (Li-Cor Biosciences, Lincoln, NE) after incubation with HRP-conjugated, Alexa680-, or IRDye800-labeled secondary antibodies (Invitrogen). Densitometry was carried out with the use of ImageJ[24].

Statistical analysis

Student's *t*-test was used to calculate differences between sensitive and insensitive cell lines. Data were expressed as mean ± SD. All *p* values are given for two-sided tests and $p \leq 0.05$ was considered significant.

RESULTS

Variability in simvastatin-induced cytotoxicity in AML cell lines

To evaluate the cause of heterogeneity in simvastatin-induced cytotoxicity, we identified AML cell lines with either a strong or a limited susceptibility to simvastatin-mediated cytotoxicity (Figure 2A). Based on an estimated EC_{50}, we divided the cell lines into two groups: sensitive ($EC_{50} \leq 5$ μM; NB4, OCI-M3 and HL60) or insensitive ($EC_{50} \geq 50$ μM; UT7-GM, TF-1 and KG1a) to simvastatin. The same division could not be made when these cell lines were exposed to the cytotoxic agent cytarabine (data not shown), e.g., KG1a was much more sensitive to cytarabine than OCI-M3. These data indicate that the difference in responsiveness to simvastatin is not a general phenomenon. In addition, no relation between responsiveness and cell cycle status or proliferation rate was observed (data not shown).

In the following experiments, the concentration of simvastatin used for the different cell lines was based on their individual EC_{50} values. First, we investigated whether mevalonate, the metabolite directly downstream of HMG-CoAR (Figure 1), was able to prevent simvastatin-induced cytotoxiticy. As depicted in Figure 2B, mevalonate prevented cytotoxicity by 91-100% in the sensitive cell lines and by 36-91% in the

insensitive cell lines. The difference between sensitive and insensitive cell lines was not statistically significant (p=0.20). In addition, inhibition of phosphorylation of ERK by simvastatin could be prevented by co-exposure to mevalonate (Figure 4D).

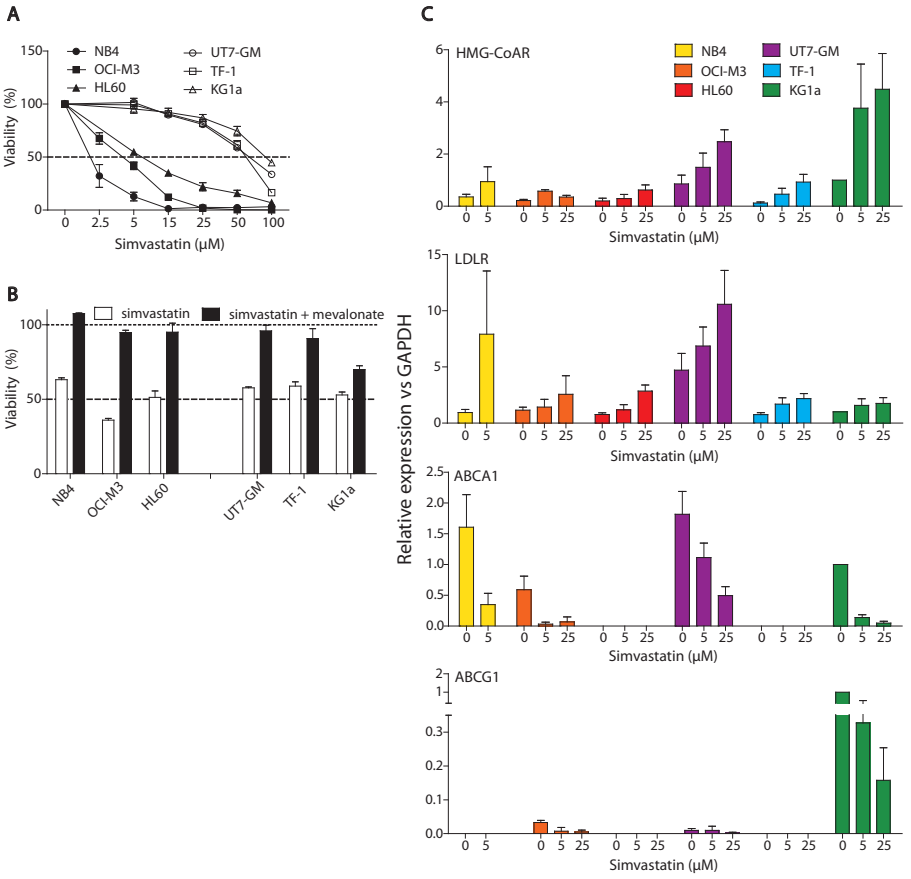


Figure 2. Mevalonate prevents simvastatin-induced cytotoxicity in differentially sensitive cell lines. (A) Cell lines were treated with 2.5 to 100 μM simvastatin or (B) with simvastatin alone (NB4: 1 μM; OCI-M3: 2.5 μM; HL60: 5 μM; UT7-GM: 50 μM; TF-1: 50 μM; KG1a: 100 μM) or in combination with mevalonate (250 μM) for 48 hours. The percentage of viable cells relative to the untreated cells was measured by a chemoluminescence assay. Cell lines are shown in order of decreasing sensitivity to simvastatin. Experiments were done at least three times in duplicate. Data are shown as mean ± SD. (C) mRNA expression of cholesterol metabolism genes in response to simvastatin. Cells were treated for 48 hours with 5 and 25 μM simvastatin. The mRNA expression levels of HMG-CoAR, LDLR, ABCA1, and ABCG1 were determined by quantitative real-time PCR and normalized to GAPDH. Cell lines are shown in order of decreasing sensitivity to simvastatin. Experiments were done at least 3 times in duplicate. Data are shown as mean ± SD.

Next, we questioned whether the differences in the degree of statin-induced cytotoxicity between the sensitive and insensitive cell lines was due to differences in biological effectiveness of simvastatin. Therefore, simvastatin-affected gene expression was studied. Genes encoding HMG-CoAR and LDLR, which mediate the first step of cholesterol synthesis and LDL-cholesterol influx, respectively, are reported to be dose-dependently upregulated upon treatment with statins[25]. ABC transporter genes ABCA1 and ABCG1, responsible for cholesterol efflux (Figure 1), are downregulated by statins in cells with significant *de novo* production of cholesterol[25]. All cell lines showed a dose-dependent increase in expression of HMG-CoAR and LDLR (Figure 2C) and, if expressed, a decrease in ABCA1 and ABCG1 mRNA expression (Figure 2C) at low doses of simvastatin. No relationship existed between basal expression levels of HMG-CoAR, LDLR, ABCA1, or ABCG1 and simvastatin-induced cytotoxicity. These observations imply that simvastatin was similarly active in all tested cell lines.

The cholesterol synthesis route is not responsible for simvastatin-induced cytotoxicity

We next established that the cholesterol synthesis route is not involved in simvastatin-induced cytotoxicity. Squalene, a cholesterol precursor produced downstream of mevalonate (Figure 1), could not prevent simvastatin-induced cytotoxicity in AML cell lines (Figure 3A). In addition, we mimicked the effects of simvastatin with ZGA, a squalene synthase inhibitor acting downstream of simvastatin in the cholesterol synthesis pathway (Figure 1). ZGA did not induce cytotoxic effects in any of the cell lines tested (Figure 3B). These results clearly indicate that inhibition of the cholesterol synthesis route within the mevalonate pathway is not critically involved in simvastatin-induced cytotoxicity.

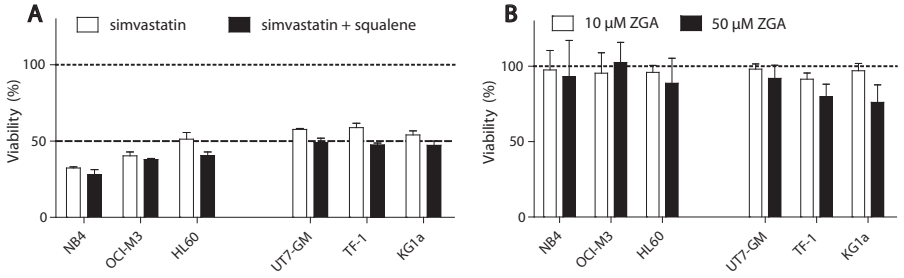


Figure 3. Differences in simvastatin response are not linked to the cholesterol synthesis route. Cell lines were treated with (A) simvastatin alone or in combination with squalene (5 µM) or with (B) ZGA (10 and 50 µM) for 48 hours. The percentage of viable cells relative to the untreated cells was measured by a chemoluminescence assay. Cell lines are shown in order of decreasing sensitivity to simvastatin. Experiments were done at least three times in duplicate. Data are shown as mean ± SD. Simvastatin concentrations: NB4: 1 µM; OCI-M3: 2.5 µM; HL60: 5 µM; UT7-GM: 50 µM; TF-1: 50 µM; KG1a: 100 µM.

GGPP and FPP prevent simvastatin-induced cytotoxicity in simvastatin-sensitive AML cell lines

To assess whether the isoprenylation pathway is involved in simvastatin-induced cytotoxicity, we firstly determined whether two compounds involved in this route, i.e., FPP and GGPP (Figure 1), are able to prevent simvastatin-induced cytotoxicity. Cytotoxicity induced by simvastatin could be partially prevented by FPP (Figure 4A,C) and to a larger extent by GGPP (Figure 4B,C). The protective effects of FPP and GGPP were more pronounced in the sensitive cell lines compared to the insensitive cell lines, with an average rescue of 56% ± 27% versus 16% ± 15% for FPP (Figure 4C; p=0.08), and 78% ± 29% versus 19% ± 17% for GGPP (p=0.04). Similarly, simvastatin-mediated phospho-ERK inhibition could be prevented by FPP, and to a larger extent by GGPP, in

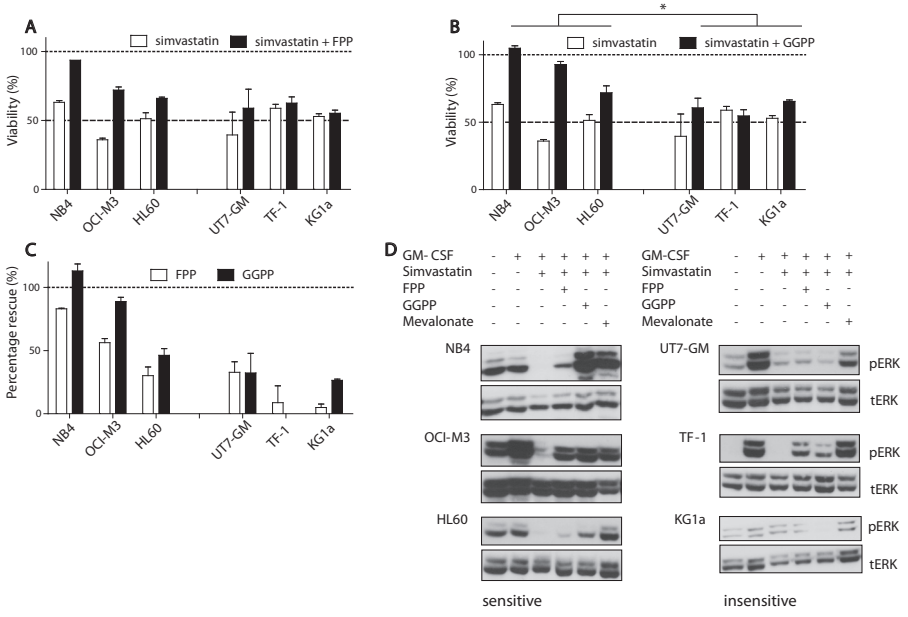


Figure 4. The effects of GGPP and FPP on simvastatin-induced cytotoxicity and phospho-ERK expression. Cell lines were treated with simvastatin alone (NB4: 1 μM; OCI-M3: 2.5 μM; HL60: 5 μM; UT7-GM: 50 μM; TF-1: 50 μM; KG1a: 100 μM) or in combination with (A) FPP (20 μM) or (B) GGPP (10 μM) for 48 hours. The percentage of viable cells relative to the untreated cells was measured by a chemoluminescence assay. Cell lines are shown in order of decreasing sensitivity to simvastatin. Experiments were done four times in duplicate. Data are shown as mean ± SD. (C) Percentage of rescue as calculated by ((average viability simvastatin + GGPP/FPP – average viability simvastatin)/(100-average viability simvastatin))×100%. (D) Cells were cultured in starvation medium (5% FCS, no GM-CSF) with simvastatin alone (NB4: 5 μM; OCI-M3: 5 μM; HL60: 5 μM; UT7-GM: 100 μM; TF-1: 100 μM; KG1a: 100 μM) or in combination with mevalonate (250 μM), FPP (20 μM), or GGPP (10 μM) for 48 hours. Cells were stimulated with GM-CSF for 15 minutes prior to harvesting. Phosphorylated (pERK) and total ERK (tERK) expression was determined by Western blot analysis. Representative Western blots of 3 experiments are shown. *p<0.05.

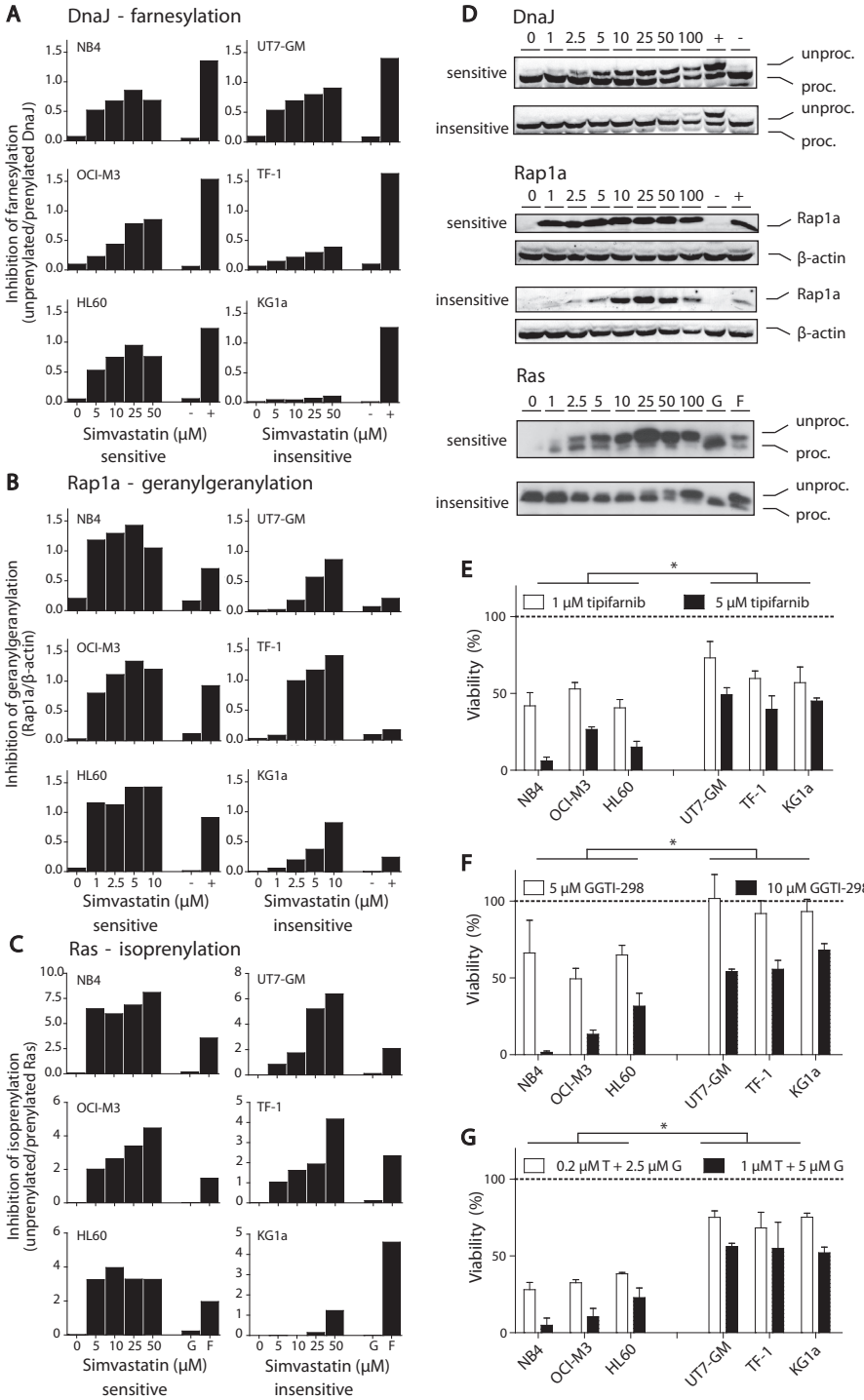
the sensitive cell lines (Figure 4D). Again, this effect was less pronounced in the insensitive cell lines. Combining GGPP and FPP slightly, but not significantly, increased the rescue compared with either GGPP or FPP alone (data not shown). These data show that the isoprenylation route is involved for simvastatin-induced cytotoxicity, and suggest that the differences in simvastatin response found between AML cell lines may reside in this route.

Differential inhibition of isoprenylation by simvastatin in AML cell lines

To analyze whether simvastatin differentially affects protein isoprenylation, we determined the effect of simvastatin on isoprenylation of DnaJ, a protein that is exclusively farnesylated[26], and of Rap1, a protein that is exclusively geranylgeranylated[27]. Simvastatin inhibited farnesylation in a dose-dependent manner in the cell lines (Figure 1, 5A). In two of the insensitive cell lines, TF-1 and KG1a, even the highest concentration of simvastatin resulted only in a minor inhibition of farnesylation. Overall, the insensitive cell lines TF-1 and KG1a showed a significant lower degree of inhibition of farnesylation ($p<0.04$) than the sensitive lines. Similarly, the inhibitory effects of tipifarnib, a specific inhibitor of FTase, on farnesylation were most pronounced in the sensitive cell lines (e.g., at 5 μM $p=0.01$). Cytotoxic effects of tipifarnib occurred at low concentrations (EC_{50} 0.2-5 μM), with the most pronounced effects on sensitive cells ($p<0.05$ at all tested concentrations; Figure 5D).

In the sensitive cell lines, geranylgeranylation was inhibited by simvastatin to a comparable degree as by GGTI-298, a specific inhibitor of GGTase, which served as a positive control (Figure 1, 5B). This inhibition of isoprenylation was already present at very low concentrations (1 μM; $p=0.002$) of simvastatin in the sensitive cell lines, whereas in the insensitive cell lines this inhibition was achieved at a 10-fold higher concentration ($p=0.02$). Interestingly, also GGTI-298 inhibited geranylgeranylation in the sensitive cell lines, but did not efficiently inhibit this in the insensitive cell lines (Figure 5B). GGTI-298 induced cytotoxicity at low concentrations in the sensitive cell lines (EC_{50} 5 μM; Figure 5E), whereas the insensitive cell lines were less sensitive to GGTI-298 (e.g., $p=0.01$ at 10 μM). When combining tipifarnib and GGTI-298, as to cover both the farnesylation and geranylgeranylation routes, we observed additive effects and more pronounced differences between the sensitive and insensitive cell lines ($p=0.003$ at 1 μM tipifarnib plus 5 μM GGTI-298; Figure 5F). These data confirm that the geranylgeranylation route and the farnesylation route are differentially affected by simvastatin in the sensitive and insensitive cell lines.

In addition, we assessed the effect of simvastatin on isoprenylation of Ras. We used an antibody directed against N-Ras, because N-Ras could be detected in all cell lines, in contrast to K-Ras and H-Ras (data not shown), and only by using this antibody a


A
DnaJ - farnesylation
Inhibition of farnesylation (unprenylated/prenylated DnaJ)
NB4
UT7-GM
OCI-M3
TF-1
HL60
KG1a
Simvastatin (μM)
sensitive
insensitive
B
Rap1a - geranylgeranylation
Inhibition of geranylgeranylation (Rap1a/β-actin)
C
Ras - isoprenylation
Inhibition of isoprenylation (unprenylated/prenylated Ras)
D
DnaJ
Rap1a
Ras
unproc.
proc.
β-actin
E
1 μM tipifarnib
5 μM tipifarnib
Viability (%)
F
5 μM GGTI-298
10 μM GGTI-298
G
0.2 μM T + 2.5 μM G
1 μM T + 5 μM G

‹ *Figure 5. Effect of simvastatin on farnesylation and geranylgeranylation.* Cells were treated with increasing concentrations of simvastatin for 48 hours. After harvesting, prenylation status of (A) DnaJ, (B) Rap1, and (C) N-Ras was determined by Western blot analysis. Ratios of unprocessed versus processed DnaJ and N-Ras and unprocessed Rap1 (Rap1a) versus β-actin are shown, as determined by densitometry with the use of ImageJ. (A) 0.2 μM tipifarnib – positive control (+); 2.5 μM GGTI-298 – negative control (-). (B) 0.2 μM tipifarnib – negative control (-); 2.5 μM GGTI-298 – positive control (+). (C) G: 2.5 μM GGTI-298; F; 0.2 μM tipifarnib. (D) Representative examples of Western blots are given of a sensitive and an insensitive cell line: for DnaJ and N-Ras, the faster migrating band represents prenylated DnaJ or N-Ras (proc(essed)), and the slower band represents unprenylated DnaJ or N-Ras (unproc(essed)). Unprenylated Rap1 levels were compared with β-actin levels. To investigate the effect of isoprenylation inhibitors on the cell lines, cells were treated with (E) tipifarnib, (F) GGTI-298, and (G) the combination of both agents for 48 hours. The percentage of viable cells relative to the untreated cells was measured by a chemoluminescence assay. Cell lines are shown in order of decreasing sensitivity to simvastatin. Data are representative of 3 independent experiments (A-D), or experiments were done at least three times in duplicate (E-G). Data are shown as mean ± SD. *p<0.05.

clear distinction could be made between prenylated and unprenylated Ras. Again, inhibition of isoprenylation in sensitive cell lines was already significant at the lowest concentration (1 μM, p=0.03), whereas the insensitive cell lines showed inhibition at higher concentrations, which was only significant at 100 μM (p=0.02).

The Ras/MEK/ERK route is differentially affected by simvastatin in AML cell lines

Rho is exclusively geranylgeranylated, whereas Ras can be both geranylgeranylated and farnesylated (Figure 1)[11,12]. To dissect these separate processes, inhibitors of the Rho route and two distinct routes downstream of Ras were explored. The ROCK inhibitor Y27632 (acting downstream of Rho) increased cytotoxicity only at high concentrations (EC_{50} 85-140 μM), and there was no difference in effects between sensitive and insensitive cell lines (Figure 6A). LY294,002, a PI3K inhibitor acting downstream of Ras, increased cytotoxicity at low concentrations (EC_{50} 14-30 μM), but no differences between sensitive and insensitive cell lines were observed (Figure 6B). The MEK inhibitor U0126 (downstream of Ras) increased cytotoxicity at low concentrations (EC_{50} 4-22 μM), and this effect was more pronounced in the sensitive cell lines (p=0.003; Figure 6C). These data together indicate that mainly the Ras/MEK/ERK route is differentially affected by simvastatin in sensitive versus insensitive cell lines, which is supported by the finding that the latter require at least a 10-fold higher simvastatin concentration for the inhibition of ERK phosphorylation (>50 μM) than the sensitive cell lines (5 μM; Figure 6D).

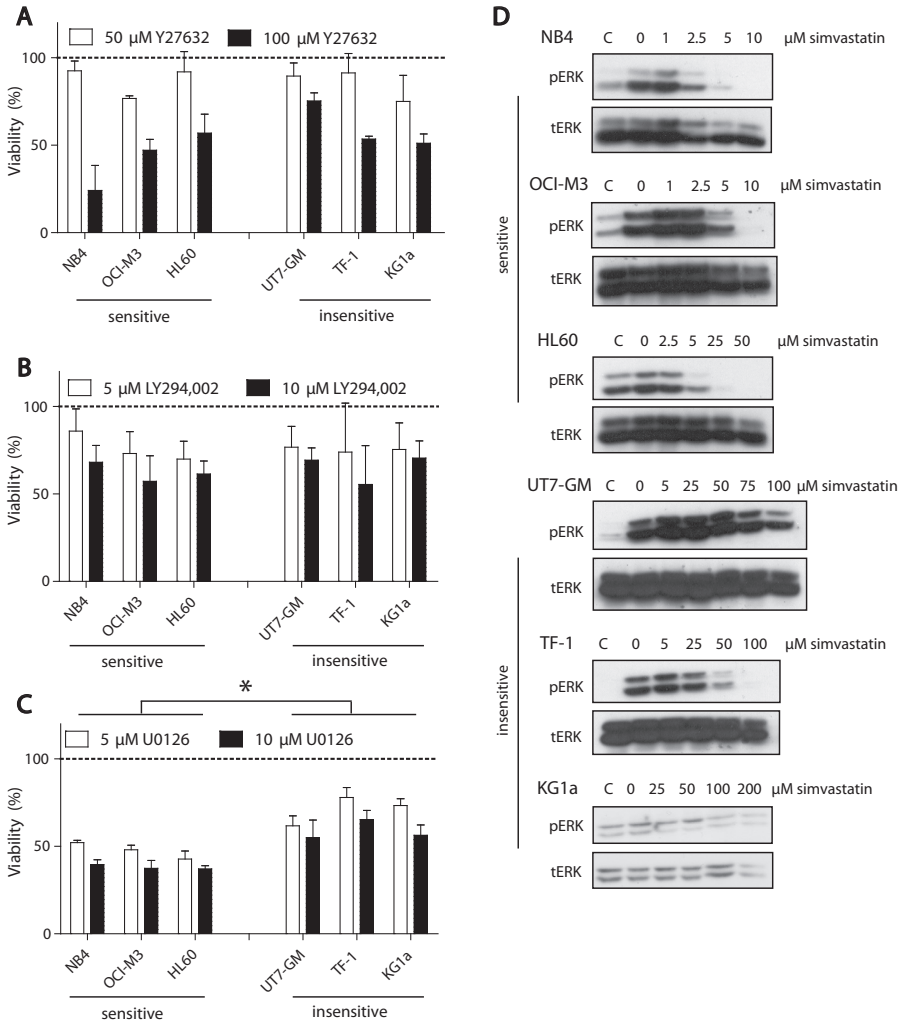


Figure 6. Effect of Uo126, Y27632 and LY294,002 on viability. Cells were treated with (A) Y27632, (B) LY294,002, and (C) Uo126 for 48 hours. The percentage of viable cells relative to the untreated cells was measured by a chemoluminescence assay. Cell lines are shown in order of decreasing sensitivity to simvastatin. Experiments were done at least three times in duplicate. Data are shown as mean ± SE. *$p<0.05$ (D) Effect of simvastatin on ERK phosphorylation. Cells were cultured in starvation medium (5% FCS, no GM-CSF) with simvastatin for 48 hours. Cells were stimulated with GM-CSF for 15 minutes prior to harvesting. Phosphorylated and unphosphorylated ERK expression were determined by Western blot analysis. Cell lines are shown in order of decreasing sensitivity to simvastatin. Representative Western blots of 3 experiments are shown. C = unstimulated control.

Simvastatin affects the isoprenylation pathway in primary human AML cells

To establish whether the mechanism of (heterogeneous) simvastatin-induced cytotoxicity in cell lines also holds true for primary human AML cells, we examined the ability of mevalonate, GGPP, and FPP to prevent simvastatin-induced cytotoxicity in these cells after 24 hours of culture. The studied AML patient group (n=11) was classified according to the French-American-British classification system as follows: M0 (n=1), M1 (n=4), M2 (n=1), M3 (n=1), M4 (n=2), and M5 (n=2). The median age of the patients was 57 years (range 35-80 years). Karyotype analysis was done in 10 of 11 patients: a normal karyotype (n=6) and an abnormal karyotype (n=4) were displayed (t(11;20), t(15;17), inv(16), and inv(3q,-7,-10)). In all patient AML cells tested, mevalonate could (partially) prevent simvastatin-induced cytotoxicity; in 57% of the samples the level of rescue exceeded 80%, whereas in the remaining samples this value varied between 25-74%. GGPP and FPP could (partially) prevent cytotoxic effects of simvastatin in 57% and 86% of the AML samples, respectively. This rescue was on average 46% (range 19-100%) when applying GGPP and 52% (range 9-100%) when FPP was used. Thus, consistent with our cell line data, we observed also in patient AML cells heterogeneity in the degree of rescue by GGPP and FPP. Maximal inhibition of geranylgeranylation and farnesylation was achieved with simvastatin concentrations similar to those used in the cell lines, but again, differences in concentrations required for this inhibition between the AML patient samples were present, ranging from 25 to >100 μM simvastatin for inhibition of farnesylation, and from 1 to 5 μM for inhibition of geranylgeranylation. These differences were also observed when treating the cells with simvastatin, tipifarnib, or GGTI-298, although higher concentrations of these agents were required to induce cytotoxicity than in the cell lines (concentration required for 25% cytotoxicity of simvastatin: EC_{25} 25-350 μM in primary AML cells versus 0.5-50 μM in cell lines; tipifarnib: 5-50 μM versus 0.04-1 μM; GGTI-298: 25-70 μM versus 2.5-10 μM).

DISCUSSION

In this study, we showed that differences in sensitivity of human AML cells to simvastatin-mediated cytotoxicity are related to differential effects of the drug on the Ras/MEK/ERK signaling pathway. These differential effects can be assigned to interference with the isoprenylation route rather than with the cholesterol synthesis route, as unambiguously demonstrated in AML cell lines, as well as in primary human AML cells. In earlier studies on differentially sensitive myeloma cells, simvastatin response appeared to be related to P-glycoprotein (P-gp) expression[28]. Indeed, some statins have been identified as substrates for multidrug resistance pumps, including

P-gp[29]. However, our study shows that expression of cholesterol metabolism genes is modulated in a similar fashion in sensitive and insensitive cell lines at low simvastatin concentrations, yet, not all cell lines were equally sensitive to cytotoxic actions of simvastatin at these concentrations. Therefore, it is unlikely that variable activity of drug efflux pumps is responsible for the observed differential effects of simvastatin.

Statin-induced cytotoxicity has been attributed to its ability to inhibit cholesterol synthesis[1,30,31]. Thus far, it has been shown that AML cells *in vitro* display an enhanced cholesterol metabolism[3-5]. Moreover, upon *in vitro* treatment with cytarabine or daunorubicin, AML cells increase their cholesterol contents, which has been suggested to protect the cells against the cytotoxic actions that are crucial for effective therapy[6]. Based on these considerations, clinical trials have been initiated to assess whether interference with the cholesterol synthesis pathway by using statins indeed promotes the efficacy of chemotherapy-mediated cell death[30,32,33]. However, in our study we show that statin-induced cytotoxicity is not caused by inhibition of cholesterol synthesis *per se*. We show that the cholesterol synthesis inhibitor ZGA, that interferes with the actions of squalene synthase, did not cause cytotoxicity in the AML cells at concentrations reported to suppress *de novo* sterol synthesis[34,35]. We also found that squalene, a cholesterol precursor, was not able to prevent simvastatin-induced cytotoxicity as did mevalonate. Together, these findings indicate that simvastatin does not exert its cytotoxic effects by blocking the cholesterol synthesis pathway. This is in agreement with results obtained with endometrial cells and squamous cell carcinoma cells[36,37], although conflicting data on the effects of cholesterol inhibition on cell survival have been published[38-40].

We show that simvastatin-induced cytotoxicity could be prevented by GGPP and FPP, indicating that simvastatin-induced cytotoxicity is likely mediated by interference with the isoprenylation route. Some reports suggest a role for protein geranylgeranylation, rather than for farnesylation, in statin-induced cytotoxicity in other (hematological) malignancies. This is in part based on the observation that the geranylgeranyltransferase inhibitor GGTI-298 was less cytotoxic than the farnesyltransferase inhibitor FTI-277, when used at comparable concentrations[11,41,42]. However, tipifarnib, the FTI used in the current study, was much more potent than GGTI-298. Moreover, we show that simvastatin does inhibit farnesylation in AML cell lines, and that simvastatin-induced cytotoxicity can partially be prevented by FPP. The reduced ability of FPP to prevent simvastatin-induced cytotoxicity compared with GGPP may be due to the production of squalene (Figure 1)[31,34,43]. The idea that FPP does not efficiently prevent simvastatin-induced cytotoxicity, because it acts as precursor of squalene, strengthens our conclusion that blockade of the cholesterol synthesis pathway is not crucial for statin-induced toxicity.

Also in primary human AML cells we observed a (partial) prevention of simvastatin-induced cytotoxicity by GGPP and FPP, as well as inhibition of isoprenylation by simvastatin at concentrations comparable with those used in cell lines. However, higher concentrations of simvastatin, tipifarnib, and GGTI-298 were required to induce cytotoxic effects. Apparently, compared to cell lines, primary AML cells are less dependent for their survival on the Ras-mediated signaling, the route we identified as being responsible for simvastatin-induced cytotoxicity.

Thus, our data indicate that inhibition of both geranylgeranylation and farnesylation by simvastatin plays a major role in statin-induced cytotoxicity in AML cells. It has been shown that Rho is exclusively geranylgeranylated[11], whereas Ras is both geranylgeranylated and farnesylated[12]. By using specific blockers of these separate routes, we were able to show that inhibitors of the Ras route, rather than inhibitors of the Rho route, mimicked the effects of simvastatin. This is in line with the finding that lovastatin-induced apoptosis can significantly be repressed by constitutive activation of the Ras/MEK/ERK pathway[44]. In addition, downregulation of the Ras/MEK/ERK pathway has been shown to potentiate statin-induced apoptosis[35]. Because Ras can be both farnesylated or geranylgeranylated, and combining FPP and GGPP does not render a higher rescue than either of the compounds alone, the Ras transduction pathway is most likely not the only pathway involved in simvastatin-induced cytotoxicity. Of course, several other proteins can become isoprenylated, e.g., Rheb proteins, nuclear lamins, Rac, and CDC42[45-47].

Sensitivity to statin-induced cytotoxicity has been reported to be cell type-dependent[48], but the present study shows that even within the same cell type, i.e., AML cells, marked differences in statin-induced cytotoxicity can be observed. This heterogeneity is in accordance with our previous report, in which we identified a differential degree of cytotoxicity within sorted primary $CD34^+$ AML samples[15]. We show that especially the activity of the Ras/MEK/ERK pathway is to a major extent responsible for differences in cytotoxicity. A 2-fold higher concentration of the ERK inhibitor U0126 was required in insensitive cell lines to induce effects comparable to those in sensitive cell lines. Likewise, we observed a relationship between simvastatin-mediated cytotoxicity and simvastatin-induced inhibition of Ras-isoprenylation as well as phospho-ERK inhibition. This difference cannot be ascribed to variation in the incidence of *RAS* mutations: the cell lines NB4, HL60, and TF-1 carry activating *RAS* mutations, whereas OCI-M3 and KG1a, do not[7,13]. In addition, it has been shown that in primary AML cells statin sensitivity is not associated with *RAS* mutations or Ras protein overexpression[7], and AML cells were shown to be much more sensitive to lovastatin than ALL cells, although the extent of *RAS* mutations was approximately equal in both

cell types[16]. So, although we found that the Ras pathway holds the key to the differences in simvastatin response between AML cell lines, the differences are not related to Ras (over)activity.

In summary, we show that cytotoxic effects of simvastatin in AML cell lines and primary human AML cells can be assigned to interference with the isoprenylation route of the mevalonate pathway, rather than with cholesterol synthesis. The differences in simvastatin-induced cytotoxic effects could, at least in part, be explained by differences in the Ras/MEK/ERK pathway within this isoprenylation route. Heterogeneity in simvastatin effects of this route was also found within AML patient samples. Our findings can potentially be useful to identify AML patients that may benefit from simvastatin co-treatment.

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Treatment with high-dose simvastatin inhibits geranylgeranylation in AML blast cells in a subset of AML patients

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ABSTRACT

It is currently unknown whether the *in vitro* effects statins display in acute myeloid leukemia (AML) cells, including lowering of cholesterol, inhibition of isoprenylation, and sensitization to chemotherapy, also occur *in vivo*. Therefore, AML mononuclear cells (MNCs) from 12 patients before and after 7 days of high-dose (7.5-15 mg/kg/day) simvastatin treatment were studied. Parallel mouse studies were performed to have, besides AML cells, also access to liver tissue, a major target of statins. Serum cholesterol levels were lowered by simvastatin in all patients, however, only limited changes in the MRNA expression of cholesterol metabolism genes were seen in patient and mouse MNCs compared to murine liver cells. Still, 29% of the patients displayed an increased *in vitro* chemosensitivity of their AML cells upon simvastatin treatment. Gene set enrichment analysis on microarray data of AML patient MNCs and $CD34^+$ cells, as well as Western blot analysis for the isoprenylated proteins DNAJ and RAP1 on murine and AML patient MNCs demonstrated that *in vivo* simvastatin treatment resulted in inhibition of geranylgeranylation in murine MNCs and in a subset of patient MNCs. In summary, our data show that simvastatin treatment results in chemosensitization and inhibition of geranylgeranylation in AML cells of a subset of patients.

INTRODUCTION

Acute myeloid leukemia (AML) is a clonal hematopoietic disease, characterized by the accumulation of immature myeloid blasts in the bone marrow. Despite intensive treatment only 5-15% of AML patients over 65 years of age can be cured, which is mainly due to the occurrence of resistance to therapeutic agents. Different mechanisms have been identified that protect AML cells against the cytotoxic effects of chemotherapy, including adaptation of cellular cholesterol homeostasis[1,2]. Cellular cholesterol levels are maintained by balanced *de novo* synthesis, influx, and efflux. Cholesterol synthesis is initiated by 3-hydroxy-3-methylglutaryl COA reductase (HMG-COAR), which is the rate-controlling enzyme of the mevalonate pathway that yields cholesterol as well as isoprenoids. The low-density lipoprotein receptor (LDLR) is responsible for cholesterol influx into cells[3]. The cholesterol efflux pumps ABCA1 and ABCG1 promote the transfer of cellular cholesterol to APOA-I and HDL, which is regulated by liver X receptor, a nuclear receptor that is activated by oxidized cholesterol derivatives (oxysterols)[4-6].

Different studies have shown that cholesterol metabolism is increased in AML cells, as reflected by high MRNA levels of HMG-COAR and LDLR, as well as increased activities of the corresponding proteins[7-9]. In addition, AML cells increase their cholesterol levels upon *in vitro* exposure to chemotherapeutic drugs, which renders these cells less susceptible to these drugs[10,11]. Consequently, a role for statins to improve standard antileukemic treatment has been suggested.

Statins inhibit HMG-COAR activity, resulting in a blockade of cholesterol synthesis, as well as inhibition of isoprenoid production. Isoprenoids are required for the attachment of small GTPases (e.g., Ras and Rho) to the plasma membrane, and their subsequent participation in signal transduction pathways that regulate growth and survival. Examples hereof are the PI3K/Akt and Ras/MEK/ERK pathways[12]. The cytotoxic effects of statins have originally been attributed to their serum cholesterol-lowering capacity[13]. However, *in vitro* studies with AML cell lines and primary AML cells have suggested that the cytotoxic effects of simvastatin are caused by a blockade of the isoprenylation route, rather than a blockade of cholesterol synthesis[14].

We aimed to verify whether the *in vitro* observed effects by statins are actually present in MNCs of AML patients treated with simvastatin. Additionally, we used a mouse model to validate our findings in more detail. This allowed us to compare the results on hematopoietic cells with liver cells, which is the major and intensively studied target organ of statins. The results show that in a subset of patients treatment with simvastatin sensitizes AML cells to *in vitro* chemotherapy. In addition, geranylgeranylation is inhibited in AML cells from simvastatin-treated patients, with only minor effects on the expression of cholesterol metabolism genes.

MATERIALS AND METHODS

Patients and patient material

Patient samples were obtained from a phase 2 feasibility study, in which AML patients were treated with high-dose simvastatin. For this study, eligible patients were those with *de novo* or relapse AML, who would receive intensive chemotherapy or palliative chemotherapy. Simvastatin was administered orally at a dosage of 7.5-15 mg/kg/day during 7 days. Eight patients were treated with 7.5 mg/kg/day simvastatin, and 4 patients received 15 mg/kg/day. On day 7 of the simvastatin treatment chemotherapy was initiated. The study was approved by the Medical Ethical Committee of the University Medical Center Groningen. All patients provided written informed consent. Bone marrow (BM) or peripheral blood (PB) samples were collected before and after 7 days of simvastatin treatment, but before chemotherapy application. The mononuclear cell (MNC) fraction was stored and thawed as described earlier[15]. Serum lipid levels were monitored with the use of standard laboratory techniques.

For additional experiments, BM or PB MNCs were used that were collected from AML patients at diagnosis after informed consent, as well as granulocyte colony-stimulating factor mobilized peripheral blood stem cells from patients eligible for autologous hematopoietic stem cell transplantation, in accordance with institutional guidelines.

Animals

As the liver is the primary target of statins, it was of interest to compare our findings in BM cells with those in liver cells. To have access to liver cells, and to extend our data on human AML BM cells, a mouse study was done. Eleven to 13 weeks old male C57Bl/6olaHsd mice were purchased from Harlan (Horst, the Netherlands) and housed under clean conventional conditions. All experimental protocols were approved by the institutional ethical committee on animal experiments. The mice were fed normal chow (RMH-B; Arie Blok, Woerden, the Netherlands), chow containing 0.1% w/w simvastatin (Merck Chemical Ltd., Nottingham, UK) for 7 days, chow containing 2% w/w WelChol (Colesevelam HCL; Daiichi Sankyo Inc., Munich, Germany) for 14 days, or the combination of the two diets (7 days WelChol, followed by 7 days WelChol + simvastatin). After treatment, a PB sample was taken. The animal was thereafter sacrificed, and liver was harvested and snap frozen. BM cells were flushed from the femurs and tibias. After standard erythrocyte lysis, nucleated cells were treated as described below. Primary rat liver cells were obtained and cultured as described by Vrenken et al[16].

Cell sorting and culture of human AML cells and murine BM cells

The patient AML MNCs were incubated with a fluorescein isothiocyanate (FITC)-conjugated antibody against CD34 (Becton Dickinson, San Jose, CA). Sorting of $CD34^+$ cells was done with the use of a MoFlo Cell Sorter (DakoCytomation, Carpinteria, CA). $CD34^+$ cells and total MNCs were cultured in LTC medium as described by van Gosliga et al[17]. All cultures were kept at 37°C and 5% CO_2.

Mouse BM cells were cultured in StemSpan (StemCell Technologies, Grenoble, France) supplemented with 10% FCS, 20 ng/mL recombinant mouse interleukin-11 (R&D Systems, Minneapolis, MN), 300 ng/mL polyethylene glycol-complexed recombinant rat stem cell factor (Amgen, Thousand Oaks, CA), and 10 ng/mL Flt3-ligand (Amgen). BM progenitor frequencies were determined by methylcellulose cultures and FACS analysis as described in the supplementary data.

Cell viability

Viability of human ($CD34^+$) AML cells and mouse BM cells was determined by the Cell Titer-Glo Luminescent cell viability assay (Promega, Madison, WI) according to the manufacturer's instructions. Plates (96-well) were prepared with 100 µL medium supplemented as described above and 10,000 cells per well. The cells were incubated with cytarabine (Mayne Pharma Benelux, Brussels, Belgium) with the use of easy load pipet tips (Greiner Bio-One, Alphen aan den Rijn, the Netherlands), and after 24 hours viability was assessed in duplicate.

Microarray experiments

Human AML $CD34^+$ and $CD34^-$ cells were sorted as indicated above, and RNA from these subfractions and from the total MNC fraction was isolated with the use of the RNeasy mini kit or the RNeasy micro kit (Qiagen, Venlo, the Netherlands), depending on the amount of sorted cells, in accordance with the manufacturer's protocol. RNA concentration, quality, and integrity were determined with the use of the Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, the Netherlands). Genome-wide expression analysis was done on Illumina BeadChip Arrays Sentrix Human-6 (46k probe sets; Illumina Inc., San Diego, CA). Typically, 0.5-1 µg was used in labeling reactions, and hybridization with the arrays was done according to the manufacturer's instructions.

Gene Set Enrichment Analysis

GSEA was done with the software package GSEA 2.0, developed by the Broad Institute of MIT and Harvard[18,19], as described elsewhere[20]. All 37,804 genes were ranked according to differential expression between pre- and post-simvastatin treatment samples based on a paired *t*-test. This ranked list of genes was compared against a large collection of

functional gene sets to determine if there was enrichment of one of these gene sets in pre- or post-treatment samples. Ranked expression data for all 37,804 genes was compared against a large collection of functional gene sets to determine if there was enrichment of one of these gene sets in pre- or post-treatment samples. A total of 156 functional genes sets as reported in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, 125 from the Biocarta database (http://www.biocarta.com), and 83 from the GenMAPP database were analysed. The statistical significance of enrichment was determined by a randomization test based on 1,000 gene permutations. For each functional set the false discovery rate (FDR) was calculated, which represents the estimated probability that a given enrichment score represents a false-positive finding. Only gene sets with an enrichment *p*-value of <0.05 and an FDR of <0.25 are reported.

Leading-edge subset analysis

The leading-edge subset is defined as the subset of genes in a functional gene set that appears in the ranked list of 37,804 genes at, or before, the point in which the running enrichment score reaches its maximum deviation from zero. The genes within this subset can be interpreted as the most important in the enrichment of the functional gene set. Leading-edge subsets were defined for all statistically enriched functional gene sets ($p<0.05$). Subsequently, overlap between leading-edge subsets from significantly enriched functional gene sets identified in the different databases was determined to discover genes belonging to more than one leading-edge subset, which might be considered key genes.

Quantitative real-time PCR

RNA was isolated from human AML MNCs, $CD34^+$, and $CD34^-$ cells, and from mouse BM and PB MNCs with the use of the RNeasy mini kit (Qiagen) and was reverse transcribed with the use of RevertAid™ H Minus M-MuLV reverse transcriptase (Fermentas, St Leon-Roth, Germany). Quantitative PCR was done with the use of the ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) as described earlier[15]. Primers and probes for the human and murine ABC transporters and HMG-CoAR and LDLR were used as described earlier[21-25]. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as endogenous control for patient material, and 18s for mouse material. Primers were obtained from Invitrogen (Breda, the Netherlands).

Western blotting

To determine DnaJ and Rap1 protein expression, cells (0.5×10^6 per mL) were cultured in the presence or absence of simvastatin (Merck Chemical Ltd.) for 24 hours. Whole cell extracts were obtained by lysing 5×10^5 cells in boiling Laemmli sample buffer

for 5 minutes. Proteins were separated by 10% or 12.5% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) in Tris-buffer with the use of a semidry electroblotter from Bio-Rad Laboratories (Veenendaal, the Netherlands). After blocking in 0.1% Tween-20 containing 5% skim powdered milk and 2% bovine serum albumin in tris-buffered saline, membranes were probed with antibodies against DnaJ (HDJ-2; Labvision, Fremont, CA), Rap1 (total), and Rap1a (unprenylated; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) according to the manufacturer's protocols. Antibody binding was visualized with enhanced chemiluminescence detection, or with the use of an Odyssey infrared scanner (Li-Cor Biosciences, Lincoln, NE) after incubation with a horseradish peroxidase (HRP)-conjugated (Dako, Glostrup, Denmark), Alexa680-, or IRDye800-labeled secondary antibody (Invitrogen). Densitometry was carried out with the use of ImageJ[26].

Statistics

The non-parametric Wilcoxon Signed Rank test was used to test whether there were differences in serum lipid concentrations, blood parameters, and gene expression between before and after treatment of AML patients with simvastatin. Student's *t*-test was used in the other analyses.

RESULTS

Treatment of AML patients with simvastatin decreases in vivo cholesterol synthesis

From 12 patients BM or PB samples before and after high-dose simvastatin treatment were available. Main characteristics of the patients are shown in Table 1. The simvastatin dosage administered was 7.5 mg/kg/day in eight patients and 15 mg/kg/day in four. To monitor the *in vivo* effectiveness of simvastatin, serum lipid levels were analyzed before and at the end of simvastatin treatment. Serum cholesterol levels were decreased after 7 days of simvastatin treatment (4.0 ± 1.0 mmol/L versus 2.3 ± 0.7 mmol/L; p=0.002). The lathosterol/cholesterol ratio (0.71 ± 0.57 versus 0.23 ± 0.21; p=0.02), which is generally used as a measure for total body cholesterol synthesis[27], was also reduced after statin treatment (Figure 1A). LDL levels were decreased as well (p=0.004), but HDL levels remained unchanged and triglycerides slightly decreased (p=0.01; Figure S1). Comparable changes in serum lipid levels were observed in patients receiving 7.5 mg/kg/day simvastatin versus 15 mg/kg/day. No distinct changes were found in the total white blood cell counts, platelet counts, or hemoglobin levels (Table 2). The blast percentages of the peripheral blood were also monitored before and after treatment, and showed no significant changes (Table 2).

Table 1. Clinical and cellular characteristics of AML patients

AML	Age (y)	Blasts* (%)	WBC (×10⁹/L)	FAB Class.	Cytogenetics	Risk group stratification†	Treatment	Simvastatin (mg/kg/day)	CD34⁺ in AML MNCs (%)
1	65	50	26.1	M5	NK	Intermediate	it	7.5	8
2	60	56	101.0	M2	NK	Intermediate	pt	7.5	36
3	72	24	17.2	M2	45,X,-X,1-13dmin; 46,XX,1-23dmin	Poor	pt	15	<1
4	23	38	3.8	M1	46,XY,5q-,9q+; 46,XY, t(5,9)	Poor	pt	7.5	23
5	82	22	1.1	M1	NK	Intermediate	pt	15	33
6	73	38	5.6	M2	NK	Intermediate	pt	15	29
7	33	80	2.4	M5	t(6;11)	Poor	pt	7.5	20
8	57	>20	1.2	M6	NK	Intermediate	it	7.5	24
9	68	20	4.8	M2	7q-	Poor	it	7.5	45
10	66	25	13.2	M6	NK	Intermediate	it	7.5	6
11	76	23	2.2	M2	NK	Intermediate	pt	15	26
12	76	80	16.8	M5	ND	ND	pt	7.5	<1

* Blasts in the bone marrow at presentation.

† Risk group stratification is based on (un)favorable cytogenetics combined with peripheral blood blast cell counts.

WBC: white blood cell counts; NK: normal karyotype; it: intensive treatments; pt: palliative treatment.

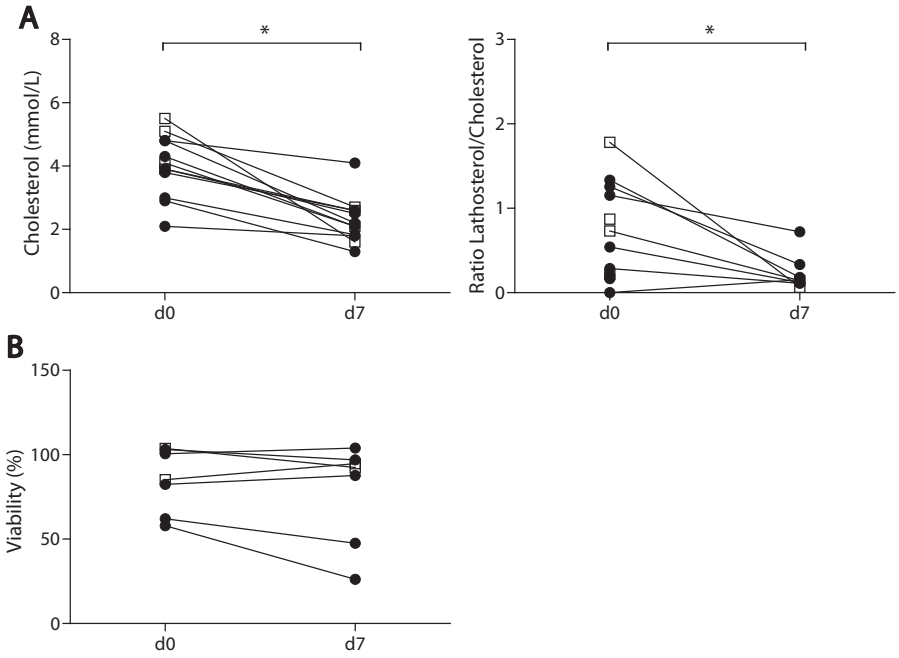

Figure 1. In vivo effects of simvastatin on serum lipid levels and in vitro chemosensitivity of AML patients. (A) Serum levels of cholesterol and the lathosterol/cholesterol ratio of patients before (do) and after 7 days (d7) of treatment with 7.5 mg/kg/day (closed circles), or 15 mg/kg/day (open squares) simvastatin. (B) Viability of sorted $CD34^+$ cells obtained from patients before (do) and after (d7) treatment with 7.5 mg/kg/day (closed circles), or 15 mg/kg/day (open squares) simvastatin upon *in vitro* treatment with 0.01 mg/mL cytarabine as shown for individual patients. *$p<0.05$.

Treatment with simvastatin increases in vitro sensitivity of sorted AML $CD34^+$ cells to chemotherapy in a subset of patients

In vitro simvastatin sensitizes primary AML cells to chemotherapy[2]. We investigated whether the AML cells exposed to simvastatin *in vivo* are more sensitive to *in vitro* chemotherapy than their untreated counterparts. Previous studies have shown that the stem cell enriched $CD34^+$ (AML) cell fraction displays higher levels of cholesterol metabolism genes[24,28], and is therefore likely more dependent on high cholesterol turnover. Therefore, we sorted $CD34^+$ AML cells from samples that were collected before and at the end of simvastatin treatment. In 7 out of 12 patients sufficient $CD34^+$ cells could be sorted for the experiments. *In vitro* culture with cytarabine for 24 hours increased cytotoxicity in 2 out of 7 AML $CD34^+$ cells exposed *in vivo* to simvastatin (Figure 1B). On average, viability of these 2 responsive samples was 60% ± 2% before and 37% ± 11% after simvastatin exposure, whereas cells of the remaining 5 patients showed 95% ± 9%

Table 2. Blood parameters of AML patients before and after simvastatin treatment

AML	WBC (×10⁹/L)		Plt (×10⁹/L)		Hb (mmol/L)		Blasts (%)	
	do	*d7*	*do*	*d7*	*do*	*d7*	*do*	*d7*
1	14.4	9.3	70	54	4.4	5.2	13	22
2	88.3	61.6	31	38	6.6	5.5	84	98
3	15.3	21.4	51	85	6.2	6.7	1	2
4	3.8	3.4	18	44	6.1	5.8	6	15
5	1.1	1.3	15	14	6.3	5.4	ND	ND
6	2.9	8.3	205	101	5.3	5.4	56	31
7	5.4	6.8	18	16	5.6	6	89	83
8	1.2	1.5	184	163	6.9	6.5	ND	ND
9	2.2	2.4	19	40	7.3	5.9	ND	ND
10	5.8	0.9	18	10	4.5	4.8	ND	ND
11	1.9	1.8	111	105	6.6	6	ND	ND
12	11.9	3.2	65	112	6.9	6	4	1

WBC: white blood cells; Plt: platelets; Hb: hemoglobin; do: before simvastatin treatment; d7: at day 7 of simvastatin treatment; ND: not determined.

viability before and 95% ± 5% after *in vivo* simvastatin (Figure 1B), indicating that only the samples that were initially sensitive to chemotherapy alone became more sensitive by simvastatin pretreatment.

Mononuclear cells of AML patients treated with simvastatin show limited changes in cholesterol metabolism gene expression

To delineate the changes that occur in AML cells upon treatment of the patients with simvastatin, microarray experiments were done on paired AML MNCs (n=10) and on paired sorted CD34⁺ AML cells (n=4; from the other patients no sufficient number of cells could be isolated), obtained before and at the end of simvastatin treatment. Treatment with simvastatin is known to result in upregulation of HMG-COAR and LDLR mRNA levels, and downregulation of ABCA1 and ABCG1 mRNA levels[29]. In the majority of patients, simvastatin treatment resulted in slight increases in HMG-COAR (6 out of 10) and LDLR (8 out of 10) mRNA levels in AML MNCs (1.3-fold ± 0.2, range 1.1-1.6-fold; and 1.8-fold ± 0.9, range 1.1-3.9-fold, respectively), whereas ABCA1 (7/10) and ABCG1 (6/10) were decreased (2.3-fold ± 1.0, range 1.2-4.1-fold; and 3.4-fold ± 1.6, range 1.7-6.1-fold, respectively; Figure 2A,C). However, these changes were not significant and not evident in all samples, despite the consistent decline in serum cholesterol levels (Figure 1A). In addition, the degree of gene expression changes in BM cells did not relate to the degree of decrease in serum cholesterol levels. In sorted CD34⁺ AML cells comparable results were obtained (Figure 2B,C). The expression levels of the tested genes were confirmed

by qPCR (Figure S2). Overall, the changes were limited and reached no statistical significance, although a trend towards a decrease of ABCA1 (p=0.07) and ABCG1 (p=0.09) could be observed in the AML MNC fraction (Figure 2A,C).

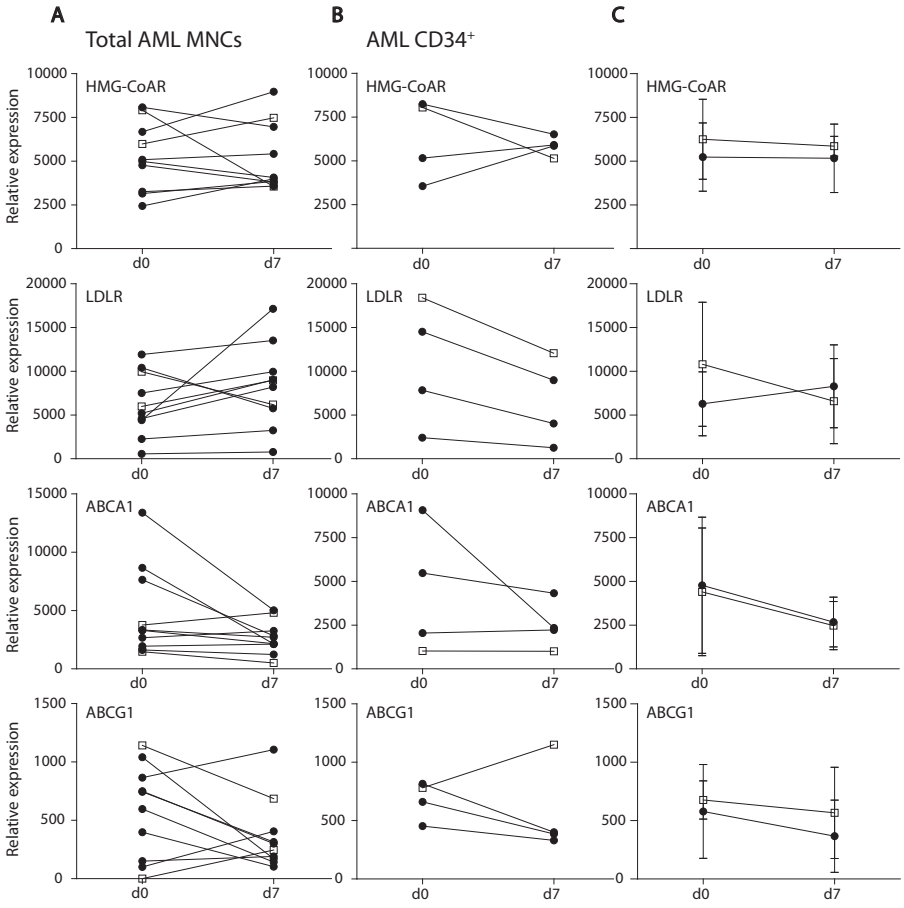

Figure 2. Cholesterol metabolism gene expression in cells of individual patients before and after simvastatin treatment, as determined by microarray analysis. Gene expression of HMG-CoAR, LDLR, ABCA1 and ABCG1 in (A) the total fraction of AML MNCs (n=10) and (B) sorted $CD34^+$ cells (n=4) before (d0) and after 7 days of treatment with 7.5 mg/kg/day (closed circles), or 15 mg/kg/day (open squares). (C) Average ± SD expression of total AML MNCs (closed circles) and $CD34^+$ cells (open squares) before and after simvastatin treatment.

Biological pathway analysis

Changes in expression at the single gene level were limited between simvastatin-treated and control AML MNCs. However, small changes in a set of genes belonging to a single biological pathway, when coordinated, can be biologically relevant. To identify such pathways affected by simvastatin treatment in AML cells, GSEA was performed (Figure 3). Using KEGG pathway definitions, GSEA identified 41 pathways enriched in simvastatin-exposed samples (Table S1), of which the most significantly enriched pathways are shown in Table 3A. No pathways were enriched in untreated samples when $p<0.05$ and false discovery rate (FDR) <0.25 were used. GSEA revealed 18 and 12 pathways enriched in simvastatin-exposed AML samples (Table 3B,C,S2,S3) according to Biocarta and GenMAPP, respectively. In simvastatin-exposed $CD34^+$ AML samples, enrichment of 12 KEGG pathways and 5 GenMAPP pathways was present, and 3 KEGG pathways were enriched in untreated $CD34^+$ AML samples (Table S4). In simvastatin-exposed $CD34^-$ AML samples, enrichment was found in 24 KEGG pathways and 13 GenMAPP pathways, and untreated $CD34^-$ AML samples showed enrichment of 3 GenMAPP pathways (Table S5).

Based on the mechanism of action of simvastatin, we expected to find pathways involved in cholesterol synthesis as well as cell signaling due to inhibition of isoprenylation of small GTPases. These pathways were indeed affected, e.g., the Rho pathway requires isoprenylated Rho and the VEGF pathway may involve isoprenylation of Ras. However, the genes that contributed to enrichment of the cholesterol (or steroid) synthesis pathway did not include HMG-COAR or LDLR, which are known to be increased *in vitro*. Instead, they included squalene epoxidase, which catalyzes the first oxygenation step of the cholesterol precursor squalene in cholesterol synthesis, and phosphomevalonate kinase, which catalyzes one of the steps resulting in the conversion of mevalonate into isopentenyl diphosphate. In addition, other metabolic

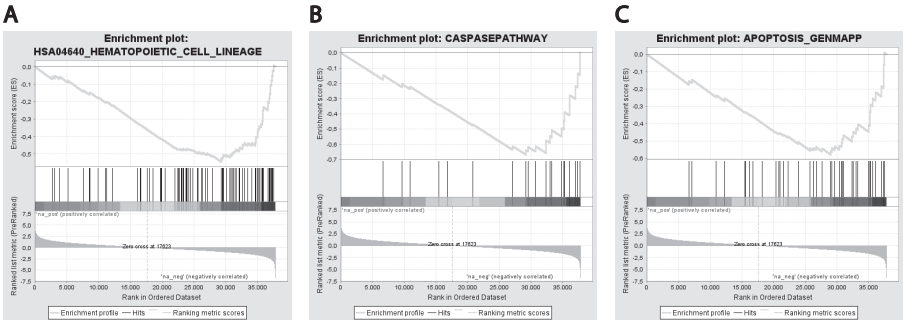

Figure 3. Examples of GSEA enrichment plots for the most enriched pathways in samples from simvastatin-treated AML patients based on (A) KEGG, (B) Biocarta, and (C) GenMAPP pathways.

Table 3. Gene sets enriched in MNCs using pathway definitions from KEGG, Biocarta, and GenMAPP with p<0.001

Pathway	*p*-value	FDR	Enriched in
KEGG			
Hematopoietic cell lineage	<0.001	0.00	post
Cell adhesion molecules	<0.001	0.00	post
Natural killer cell mediated cytotoxicity	<0.001	0.00	post
Type I diabetes mellitus	<0.001	0.00	post
T cell receptor signaling pathway	<0.001	0.00	post
Biosynthesis of steroids	<0.001	0.01	post
VEGF signaling pathway	<0.001	0.01	post
Antigen processing and presentation	<0.001	0.01	post
Cytokine cytokine receptor interaction	<0.001	0.02	post
Wnt signaling pathway	<0.001	0.02	post
MAPK signaling pathway	<0.001	0.05	post
Calcium signaling pathway	<0.001	0.05	post
Regulation of actin cytoskeleton	<0.001	0.11	post
Biocarta			
Caspase pathway	<0.001	0.01	post
Chemical pathway	<0.001	0.01	post
TCR pathway	<0.001	0.01	post
GPCR pathway	<0.001	0.02	post
Rho pathway	<0.001	0.02	post
HIVNef pathway	<0.001	0.05	post
GenMAPP			
Apoptosis GenMAPP	<0.001	0.00	post
Apoptosis	<0.001	0.00	post
Apoptosis KEGG	<0.001	0.00	post
Pyruvate metabolism	<0.001	0.00	post
Propanoate metabolism	<0.001	0.01	post
Calcium regulation in cardiac cells	<0.001	0.06	post

pathways requiring acetyl-CoA, and pathways involved in immune responses were enriched. With the exception of the cholesterol biosynthesis pathway, the pathways identified in the total AML MNC fraction were distinct from those identified in the AML $CD34^+$ fraction, suggesting that the effects of simvastatin can differ between primitive and mature cells. Leading edge analysis revealed key regulatory genes common to the

identified pathways, such as AKT1 and MAPK3 (ERK1) for KEGG pathways, and PRKCB1 and PRKCA for Biocarta (Table s6), suggesting that isoprenylation-dependent signaling may have been affected.

Differential effects of simvastatin on cholesterol metabolism gene expression in murine bone marrow and liver cells

Next, we questioned whether absence of changes in cholesterol metabolism genes in MNCs despite the physiological decrease of serum cholesterol levels reflects cell type-dependent effects. Therefore, a mouse model was used, which enabled to make a distinction between the effects of simvastatin on hematopoietic cells and liver cells, the latter being the major source of cholesterol production. Mice treated with simvastatin or simvastatin in combination with WelChol, to mimic the increased cholesterol metabolism in AML cells, displayed no different cell counts and BM and PB composition compared with their untreated counterparts (Table s7). BM progenitor frequencies, as determined by FACS analysis and CFC assay, remained unchanged, although a slight increase in LSK number (6.3 ± 0.5% to 8.3 ± 1.4%; p=0.03) upon simvastatin treatment was observed (Figure s3).

In addition, we compared changes in expression of cholesterol metabolism genes in murine BM MNCs with liver cells upon *in vivo* treatment with simvastatin. In liver cells, simvastatin induced LDLR and HMG-CoAR mRNA expression by 2- (p=0.02) and 12-fold (p<0.001), respectively, whereas no changes in ABCA1 and ABCG1 expression were noticed (Figure 4). BM MNCs showed no changes in LDLR and HMG-CoAR expression upon simvastatin treatment. In contrast to liver cells, ABCA1 and ABCG1 mRNA expression was reduced in simvastatin-treated MNCs (1.6-fold (p=0.004) and 2.1-fold (p=0.005), respectively). Pretreatment with WelChol, to induce cholesterol synthesis, increased the expression of LDLR 2-fold (p=0.009), and HMG-CoAR was 8-fold increased (p<0.001) in liver cells. HMG-CoAR increased even 47-fold in these cells when WelChol treatment was combined with simvastatin (p=0.003). In contrast, treatment with both WelChol and simvastatin did not affect the expression of cholesterol metabolism genes in the BM MNC. In addition, the expression in hematopoietic cells that are disconnected from the BM microenvironment (i.e., PB MNCs) was not affected by simvastatin treatment (Figure 4).

Simvastatin treatment of patients and mice inhibits geranylgeranylation in bone marrow cells

An alternative way in which statins might affect BM MNCs is by inhibition of farnesylation and/or geranylgeranylation. This would be in line with our GSEA data, demonstrating that simvastatin affected pathways involved in signal transduction (Table 3, s1-3) that

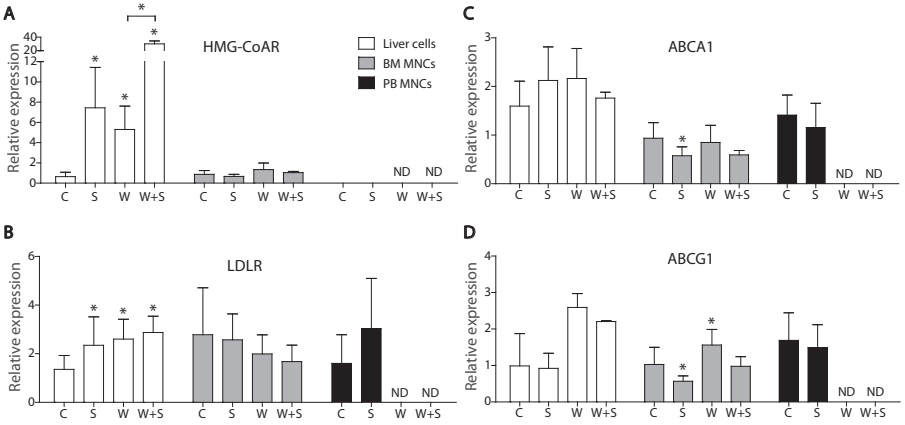

Figure 4. mRNA expression of cholesterol metabolism genes in liver cells, bone marrow (BM) MNCs and peripheral blood (PB) MNCs of mice after in vivo simvastatin treatment. mRNA expression of (A) HMG-CoA reductase; (B) LDL receptor; (C) ABCA1; and (D) ABCG1 was determined in BM, PB, and liver cells of mice that were fed with a diet containing 0.1% w/w simvastatin (S), 2% w/w welchol (W), or both (W+S). C: control chow fed mice. Mean ± SD is shown for 11 (control and simvastatin), or 3 (welchol and simvastatin + welchol) mice per group. 18S served as a housekeeping gene. Expression is shown as the relative expression versus 18S, with the first control set at one. ND: not determined. *p<0.05.

often require isoprenylation of upstream regulators. Therefore, we determined whether *in vivo* simvastatin treatment of mice also resulted in inhibition of isoprenylation. No inhibition of farnesylation was observed in mouse BM MNCs and liver cells upon *in vivo* treatment with simvastatin. However, *in vivo* treatment of mice with simvastatin resulted in differential effects on geranylgeranylation in BM and liver cells: simvastatin increased unprocessed Rap1 levels, a protein that is exclusively geranylgeranylated, in BM MNCs (Figure 5A), which could not be demonstrated in liver cells (Figure 5B). In addition, in 1 of the 4 AML patients tested, inhibition of geranylgeranylation was detected (Figure 5C), whereas no inhibition of farnesylation was observed. The limited available material from the patients that showed *in vitro* chemosensitization by simvastatin treatment precluded to assess the isoprenylation status in these patients' BM cells.

Inhibition of geranylgeranylation occurs at low concentrations of simvastatin

Next, we analyzed whether the limited effects of treatment with simvastatin on cholesterol metabolism gene expression in BM MNCs may be related to the relative low concentration achieved in the BM compartment. *In vitro* treatment of primary human and mouse BM MNCs showed that effects on cell survival, cholesterol metabolism gene expression, and the degree of farnesylation were pronounced only at high

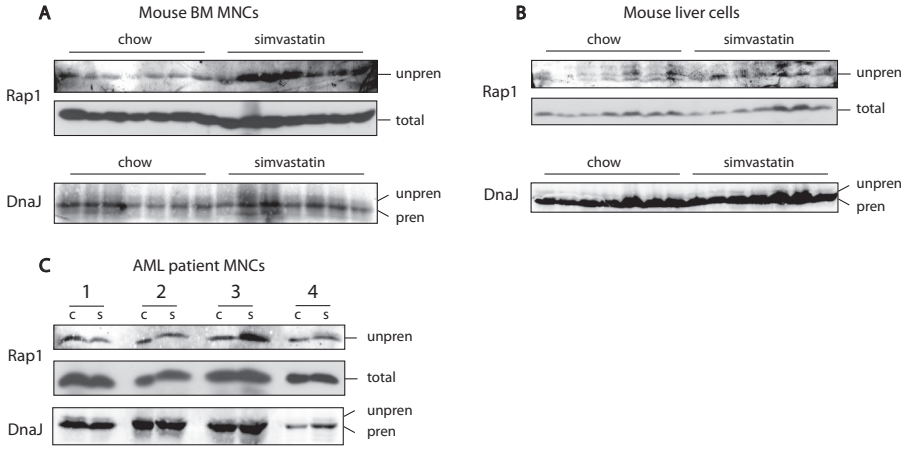

Figure 5. Inhibition of isoprenylation upon in vivo treatment with simvastatin. (A,B) Inhibition of geranylgeranylation of Rap1 and of farnesylation of DnaJ in control mice (chow) and mice treated for 7 days with 0.1% w/w simvastatin in (A) BM MNCs and (B) liver cells. Unprenylated (unpren) and total levels of Rap1 are shown, as well as unprenylated and prenylated (pren) levels of DnaJ for 7 mice in each group. (C) Prenylation status of MNCs from four AML patients before (c) and after 7 days of simvastatin treatment (s).

concentrations that are not likely to be achieved *in vivo* (>5 μM)[30] when incubated for 24 hours (Figure S4, 6A). Inhibition of Rap1 geranylgeranylation, however, occurred at concentrations as low as 0.2 μM simvastatin in the culture medium, and was maximal at 1-5 μM in both mouse and human normal BM MNCs (Figure 6B). In contrast, in primary hepatocytes inhibition of geranylgeranylation was observed only at concentrations of 25 μM or higher (Figure 6B). These data show that, although at these concentrations all theoretically predicted effects of simvastatin can occur, only inhibition of geranylgeranylation takes places at low, *in vivo* achievable concentrations.

DISCUSSION

The present study is the first to demonstrate that treatment of AML patients with high-dose simvastatin inhibits isoprenylation in their MNCs, but does not affect the expression of cholesterol metabolism genes in these cells, despite a reduction in serum cholesterol levels. This striking finding provides a novel insight in the mechanism behind statin-induced effects in normal and leukemic hematopoietic cells.

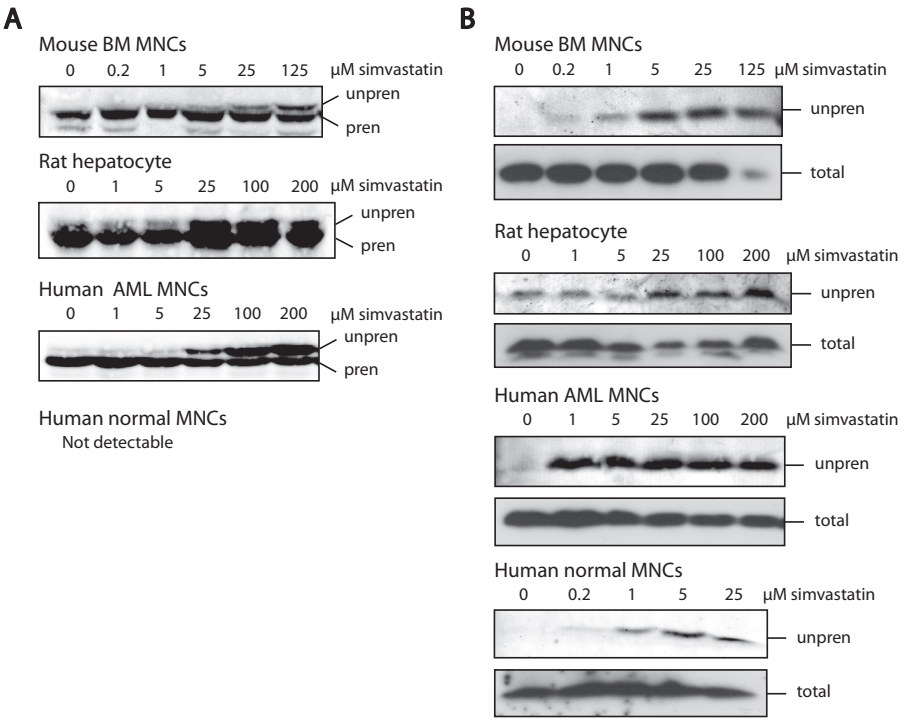

Figure 6. In vitro effects of simvastatin on prenylation status. Inhibition of farnesylation of (A) DnaJ and geranylgeranylation of (B) Rap1 after 24 hours of treatment with simvastatin in primary mouse BM MNCs, rat hepatocytes, human AML MNCs and normal human MNCs. Representative blots of at least 3 experiments are shown. unpren: unprenylated protein; pren: prenylated protein.

Statin treatment resulted in an increased *in vitro* sensitivity of AML CD34$^+$ cells to chemotherapy in about one third of the patients. These findings extend *in vitro* data demonstrating that AML cells display an increased sensitivity to chemotherapy upon statin treatment[10,11]. Differences in response to chemotherapy combined with statins were reported before in a clinical study with pravastatin combined with idarubicin and high-dose ARA-C[13]. These differences were associated with the occurrence of cholesterol rebounds after the initial decrease of cholesterol levels, but can also be due to other interpatient differences in cholesterol metabolism[13,31]. However, in our study chemosensitivity was not related to basal or simvastatin-affected expression levels of cholesterol metabolism genes. Only AML samples that are *in vitro* sensitive to chemotherapy became more sensitive when *in vivo* pretreated with simvastatin. This is in line with a former report showing that *in vitro* combination treatment with lovastatin

and daunorubicin or cytarabine was only more effective in AML samples that displayed sensitivity to chemotherapy alone[32].

Simvastatin treatment of the AML patients resulted in a significant decline in serum cholesterol levels, reflecting a decreased cholesterol synthesis by the liver. We observed indeed that cholesterol metabolism gene expression was affected in mouse liver cells by statin treatment. However, in the total or $CD34^+$ cell fraction from AML patients who underwent simvastatin treatment only limited effects were found on the MRNA expression of single cholesterol metabolism genes. This challenges previous *in vitro* reports describing that statins predominantly affect cholesterol metabolism in AML cells[2,10]. Apparently, simvastatin-mediated effects are dependent on the cell type investigated, which was already suggested by *in vitro* data showing varying sensitivities of different cancer cell lines to statin treatment[33]. Alternatively, the plasma concentration of simvastatin might not be sufficiently high due to low bioavailability[34,35]. Peak plasma levels upon treatment with 4 mg/kg/day lovastatin, a statin that is pharmacokinetically comparable to simvastatin[35,36], ranged from 0.1-3.9 μM[30]. With *in vitro* experiments, we showed that this concentration has limited effects on the expression of cholesterol metabolism genes in AML cells.

Studying the expression of single genes did not reveal clear changes upon simvastatin treatment. However, subtle, but coordinated changes in gene expression within a certain pathway can have significant biological effects. With GSEA, we showed that signaling pathways were affected by simvastatin treatment of which some participants require isoprenylation. This observation elaborates on the previous finding that the synthesis of isoprenoids, products of the mevalonate pathway, is inhibited by statins *in vitro*[14,37]. Prenylated proteins mainly belong to the superfamily of RAS-GTPases, which are involved in a number of cellular processes, including cell signaling, cell differentiation and proliferation[38]. In many AML patients RAS (predominantly N-RAS) is constitutively active due to mutations or autocrine production of growth factors[39,40]. Interestingly, *in vitro* data indicate that RAS signaling can be affected by statins, which results in inhibition of a number of downstream signaling pathways, including the MEK/ERK pathway[29,41,42].

It appeared that simvastatin mainly inhibited geranylgeranylation in AML cells of simvastatin-treated patients. Our *in vitro* data reflect these findings, as we observed inhibition of geranylgeranylation in AML MNCS and mouse BM cells at low simvastatin concentrations, whereas other effects, e.g., inhibition of farnesylation or changes in cholesterol metabolism genes, could only be observed at concentrations that were 25- to 100-fold higher. These high concentrations are also required to inhibit ERK phosphorylation, and to induce cytotoxic effects[14]. As such high simvastatin levels cannot be achieved *in vivo*[30], it is unlikely that *in vivo* treatment with statins will be effective

in totally blocking RAS/MEK/ERK signaling. For future research it would be attractive to combine statins with tipifarnib, a farnesyltransferase inhibitor, since the combined use of these agents additively blocked ERK-signaling in AML cells *in vitro*[15], and might affect both farnesylation and geranylgeranylation at clinical achievable concentrations. However, the RAS-signaling pathway is not the only pathway affected by statins, as demonstrated by GSEA. Therefore, other affected pathways may coordinately modulate cellular properties of AML cells and contribute to (clinical) effects.

Inhibition of geranylgeranylation, as well as chemosensitization was not found in all patients. Our previous *in vitro* data showed that within different patient samples a heterogeneity in response to simvastatin exists[14,15]. Not surprisingly, this also holds true for the clinical setting, and may be even more influenced by interpatient differences that do not read out *in vitro*, such as varying simvastatin plasma levels due to variation in for example CYP3A4 activity[31].

GSEA on the total AML MNC fraction also revealed pathways involved in immune signaling to be affected by simvastatin. These findings challenge to interpret the studies in AML patients that underwent allogeneic stem cell transplantation differently. Here, a reduced incidence of graft versus host disease was observed when the donors had been using low-dose simvastatin[43]. These effects of simvastatin may be attributed to their effect on prenylated proteins (e.g., RAS, RAC, RHO, RAP1, and CDC42), which are indeed involved in immune function[44]. In addition, statins are also able to block lymphocyte function-associated antigen-1 (LFA-1), and thereby T cell adhesion, activation, and proliferation in a HMG-COAR-independent way[45,46], which may have attributed to the effects on the immune pathways as well.

In summary, the present study demonstrates that treatment of AML patients with simvastatin inhibits geranylgeranylation in a subset of patients, in the absence of marked changes in cholesterol metabolism gene expression in AML MNCS. This finding breaks with the conventional idea that inhibition of cholesterol synthesis does the trick in the treatment of AML patients with simvastatin. Our findings encourage further research to exploit statin-induced inhibition of geranylgeranylation in AML patients.

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SUPPLEMENTARY DATA

Materials and methods - mouse stem cell and progenitor assays

To quantify the number of mouse progenitors after diet, the number of colony-forming units-granulocyte/macrophage (CFU-GM) was determined with the use of standard methylcellulose cultures (0.8% methylcellulose (Sigma-Aldrich), 30% FCS in α-MEM (StemCell Technologies)) supplemented with 20 ng/mL recombinant murine granulocyte-macrophage colony-stimulating factor (rmGM-SCF; R&D systems) and 100 ng/mL polyethylene glycol-complexed recombinant rat stem cell factor (peg rrSCF; Amgen). Cells were cultured at 37°C and 5% CO_2, and counted on day 7 with the use of an inverted microscope.

Stem cell and progenitor frequencies were determined by FACS-analysis on the basis of the combinatorial expression of cell surface antigens. Cells were stained with a panel of biotin-conjugated lineage-specific antibodies (containing antibodies to CD3e, CD11b (Mac1), CD45R/B220, Gr-1 (Ly-6G and Ly-6C), and TER-119 (Ly-76)), phycoerythrin (PE)-conjugated sca-1, allophycocyanin (APC)-conjugated antibody to c-Kit, PacificBlue-conjugated antibody to CD34, and PE-Cy7-conjugated antibody to CD16/32. After being washed, cells were incubated with streptavidin-APC-Cy7. All antibodies were purchased from BD Biosciences. The fluorescence activated cell-sorting analyses were done on a LSR-II (BD Biosciences). Lineage-depleted (Lin^-) BM cells were defined as the 5% of cells showing the least APC-Cy7 intensity. Hematopoietic stem cells (HSCs) were defined as $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ and progenitors as $Lin^-Sca\text{-}1^-c\text{-}Kit^+$. In addition, the progenitor cell fraction was separated into common myeloid progenitors (CMP; $Lin^-Sca\text{-}1^+c\text{-}Kit^+CD34^+CD16/32^{lo}$), granulocyte-macrophage progenitors (GMP; $Lin^-Sca\text{-}1^+c\text{-}Kit^+CD34^+CD16/32^+$), and megakaryocyte-erythroid progenitors (MEP; $Lin^-Sca\text{-}1^+c\text{-}Kit^+CD34^-CD16/32^-$). Data were analyzed with the use of FlowJo (Tree Star, Ashland, OR) software.

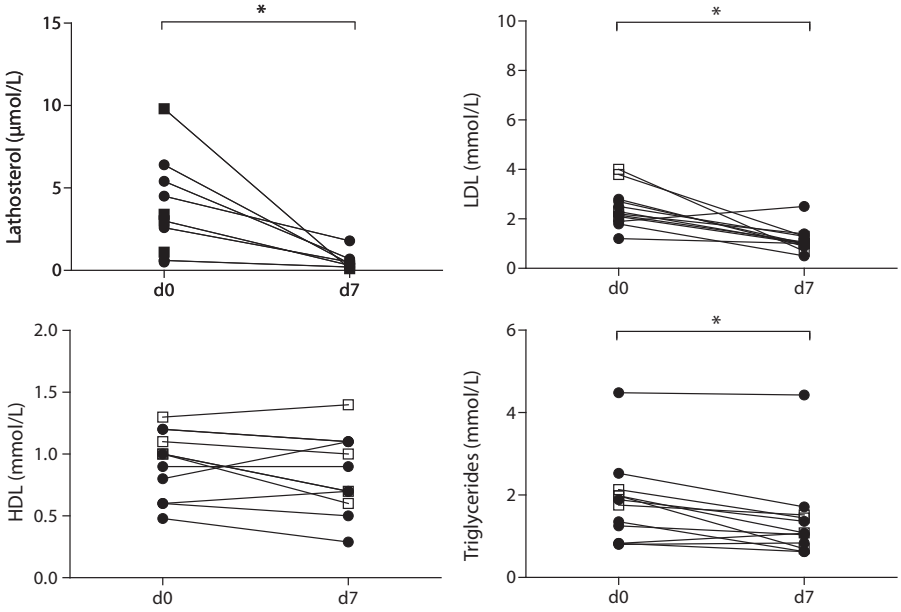


Figure S1. In vivo effects of simvastatin on serum lipid levels and in vitro chemosensitivity of AML patients. Serum levels of lathosterol, LDL, HDL, and triglycerides of patients before (do) and after 7 days (d7) of treatment with 7.5 mg/kg/day (closed circles) or 15 mg/kg/day (open squares) simvastatin. *$p<0.05$.

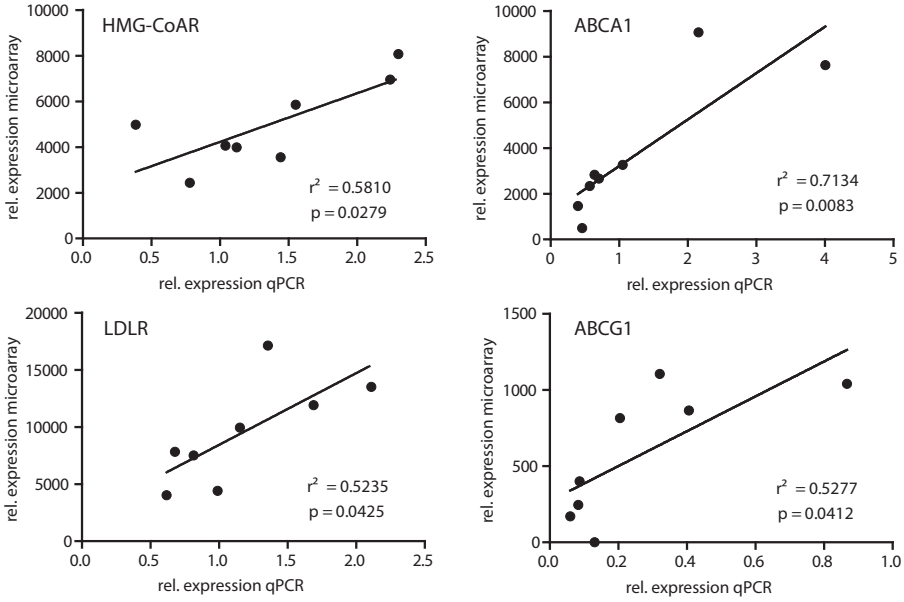


Figure S2. Verification of microarray expression data of cholesterol metabolism genes by quantitative RT-PCR. Expression of HMG-CoAR, LDLR, ABCA1, and ABCG1 in 3 total MNC fractions and one $CD34^+$ cell fraction, as determined by microarray analysis, was compared with qPCR expression levels by linear regression.

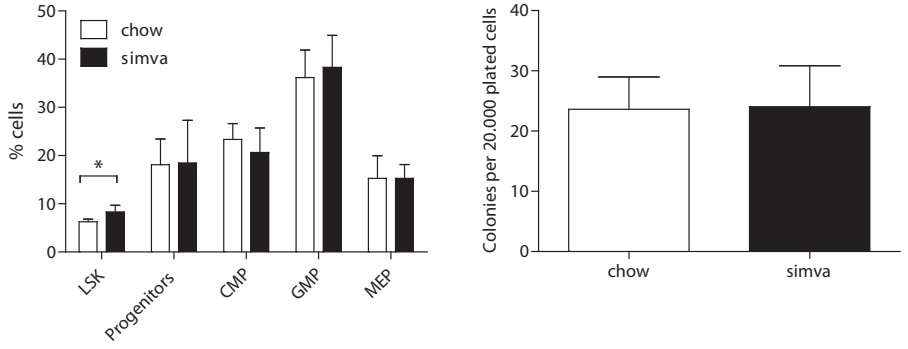


Figure S3. In vivo effects of simvastatin on progenitor frequencies of mouse BM cells. (A) FACS analysis for stem cells and progenitors and (B) a colony forming assay using BM MNCs from control mice (chow) and from mice treated with 0.1% w/w simvastatin (simva). LSK and progenitors are shown as a percentage within the 5% lineage-negative cells; CMP, GMP and MEP are shown as a percentage of the total progenitor fraction. Mean ± SD is shown for 4 control mice and 5 simvastatin-fed mice. *p<0.05.

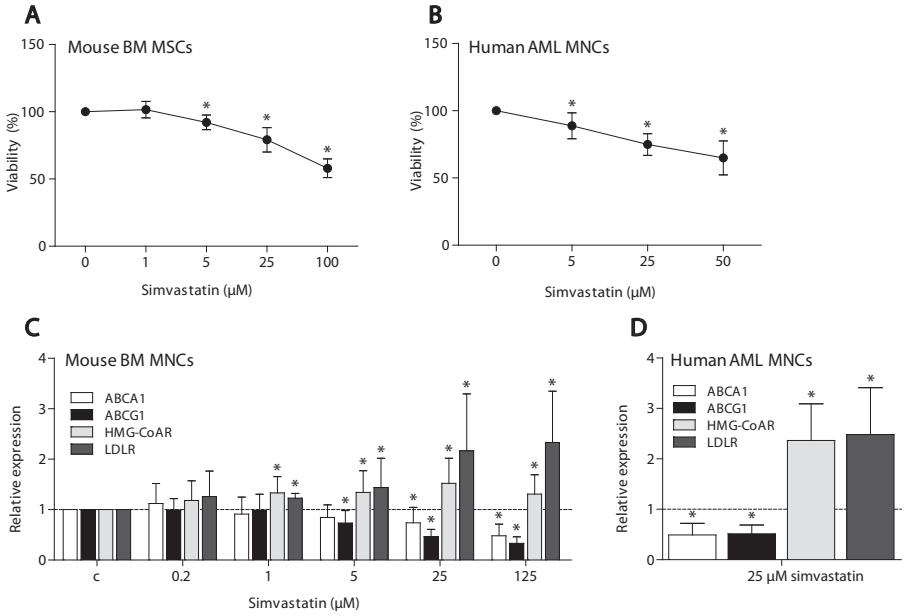


Figure S4. In vitro effects of simvastatin cell viability and gene expression. Cell viability of (A) mouse BM and (B) human AML MNCs upon treatment with different simvastatin-concentrations for 24 hours. mRNA expression of cholesterol metabolism genes in (C) mouse BM and (D) human AML MNCs upon simvastatin treatment. Control was set at 1. 18S (mouse) or GAPDH (human) served as a housekeeping gene. Data are shown as mean ± SD of n=7 (A), n=12 (B), n=8 (C) and n=6 (D). *p<0.05 versus control.

Table S1. Results of gene set enrichment analysis of MNCs using pathway definitions from KEGG

Pathway	*p*-value	FDR	Enriched in
Hematopoietic cell lineage	<0.001	0.00	post
Cell adhesion molecules	<0.001	0.00	post
Natural killer cell mediated cytotoxicity	<0.001	0.00	post
Type I diabetes mellitus	<0.001	0.00	post
T cell receptor signaling pathway	<0.001	0.00	post
Biosynthesis of steroids	<0.001	0.01	post
VEGF signaling pathway	<0.001	0.01	post
Antigen processing and presentation	<0.001	0.01	post
Cytokine cytokine receptor interaction	<0.001	0.02	post
Wnt signaling pathway	<0.001	0.02	post
MAPK signaling pathway	<0.001	0.05	post
Calcium signaling pathway	<0.001	0.05	post
Regulation of actin cytoskeleton	<0.001	0.11	post
Focal adhesion	0.001	0.06	post
Leukocyte transendothelial migration	0.001	0.05	post
JAK STAT signaling pathway	0.001	0.07	post
Tight junction	0.001	0.06	post
Axon guidance	0.002	0.01	post
Small cell lung cancer	0.002	0.05	post
Pathogenic Escherichia coli infection EPEC	0.002	0.04	post
B cell receptor signaling pathway	0.002	0.04	post
Toll like receptor signaling pathway	0.003	0.06	post
Pancreatic cancer	0.003	0.06	post
Long term potentiation	0.005	0.05	post
Fc epsilon RI signaling pathway	0.005	0.05	post
Pyruvate metabolism	0.005	0.04	post
Pathogenic Escherichia coli infection EHEC	0.006	0.04	post
Fatty acid metabolism	0.010	0.06	post
Adipocytokine signaling pathway	0.011	0.11	post
Glioma	0.012	0.12	post
Benzoate degradation via CoA ligation	0.012	0.05	post
Apoptosis	0.013	0.11	post
Arachidonic acid metabolism	0.018	0.11	post
Neuroactive ligand receptor interaction	0.022	0.26	post
Renal cell carcinoma	0.027	0.16	post
TGF beta signaling pathway	0.028	0.18	post
PPAR signaling pathway	0.031	0.14	post
Glycolysis and gluconeogenesis	0.034	0.16	post
Propanoate metabolism	0.036	0.12	post
Adherens junction	0.037	0.18	post
ECM receptor interaction	0.044	0.18	post
Pentose and glucuronate interconversions	0.046	0.13	post

Table S2. Results of gene set enrichment analysis of MNCs using pathway definitions from Biocarta

Pathway	*p*-value	FDR	Enriched in
Caspase pathway	<0.001	0.01	post
Chemical pathway	<0.001	0.01	post
TCR pathway	<0.001	0.01	post
GPCR pathway	<0.001	0.02	post
Rho pathway	<0.001	0.02	post
HIVNef pathway	<0.001	0.05	post
BCR pathway	0.002	0.02	post
Calcineurin pathway	0.002	0.01	post
P53hypoxia pathway	0.002	0.04	post
CREB pathway	0.003	0.03	post
Biopeptides pathway	0.003	0.05	post
Fas pathway	0.003	0.05	post
AMI pathway	0.003	0.05	post
CTLA4 pathway	0.003	0.03	post
NOS1 pathway	0.005	0.04	post
Csk pathway	0.007	0.04	post
Mitochondria pathway	0.007	0.04	post
Death pathway	0.008	0.05	post
VIP pathway	0.009	0.06	post
FceR1 pathway	0.010	0.08	post
PAR1 pathway	0.012	0.07	post
NKcells pathway	0.012	0.05	post
GATA3 pathway	0.020	0.08	post
TEL pathway	0.021	0.08	post
Stem pathway	0.022	0.07	post
Ceramide pathway	0.023	0.08	post
Erk pathway	0.025	0.09	post
IL12 pathway	0.025	0.08	post
ECM pathway	0.025	0.11	post
PGC1a pathway	0.027	0.11	post
PDGF pathway	0.029	0.14	post
PPARa pathway	0.030	0.13	post
NO2IL12 pathway	0.031	0.10	post
MEF2D pathway	0.033	0.13	post
SppA pathway	0.038	0.15	post
ATM pathway	0.046	0.15	post
NDKdynamin pathway	0.047	0.18	post

Table S3. Results of gene set enrichment analysis of MNCs using pathway definitions from GenMAPP

Pathway	*p*-value	FDR	Enriched in
Apoptosis GenMAPP	<0.001	0.00	post
Apoptosis	<0.001	0.00	post
Apoptosis KEGG	<0.001	0.00	post
Pyruvate metabolism	<0.001	0.00	post
Propanoate metabolism	<0.001	0.01	post
Calcium regulation in cardiac cells	<0.001	0.06	post
GPCRDB class A rhodopsin like	0.001	0.12	post
Smooth muscle contraction	0.002	0.09	post
Peptide GPCRs	0.003	0.06	post
Mitochondrial fatty acid betaoxidation	0.004	0.01	post
Cholesterol biosynthesis	0.005	0.03	post
Glycolysis	0.011	0.11	post
Glycolysis and gluconeogenesis	0.012	0.11	post
Gluconeogenesis	0.013	0.10	post
Valine leucine and isoleucine degradation	0.019	0.10	post
Integrin mediated cell adhesion KEGG	0.030	0.17	post
Lysine degradation	0.035	0.15	post
Citrate cycle TCA cycle	0.049	0.13	post

Table S4. Results of gene set enrichment analysis of CD34⁺ cells using pathway definitions from KEGG and GenMAPP

Pathway	*p*-value	FDR	Enriched in
KEGG			
Regulation of autophagy	0.005	0.10	pre
Glycan structures degradation	0.011	0.10	pre
N glycan degradation	0.018	0.10	pre
Ribosome	<0.001	0.02	post
Selenoamino acid metabolism	0.008	0.16	post
Androgen and estrogen metabolism	0.003	0.17	post
Valine leucine and isoleucine degradation	0.005	0.23	post
Cysteine metabolism	0.028	0.21	post
Methionine metabolism	0.020	0.20	post
Aminophosphonate metabolism	0.021	0.18	post
Nitrogen metabolism	0.026	0.22	post
Histidine metabolism	0.028	0.20	post
Purine metabolism	0.004	0.21	post
Ubiquitin mediated proteolysis	0.020	0.21	post
Naphthalene and anthracene degradation	0.027	0.21	post
GenMAPP			
Ribosomal proteins	<0.001	0.00	post
Cholesterol biosynthesis	0.008	0.16	post
Ubiquitin mediated proteolysis	0.003	0.15	post
Nitrogen metabolism	0.023	0.24	post
Nuclear receptors	0.014	0.19	post

Table S5. Results of gene set enrichment analysis of $CD34^-$ cells using pathway definitions from KEGG and GenMAPP

Pathway	*p*-value	FDR	Enriched in
KEGG			
T cell receptor signaling pathway	<0.001	0.01	post
Antigen processing and presentation	<0.001	0.02	post
Natural killer cell mediated cytotoxicity	<0.001	0.08	post
Regulation of actin cytoskeleton	<0.001	0.08	post
Cytokine cytokine receptor interaction	<0.001	0.11	post
Focal adhesion	0.001	0.20	post
JAK STAT signaling pathway	0.003	0.14	post
Tight junction	0.003	0.16	post
VEGF signaling pathway	0.003	0.11	post
B cell receptor signaling pathway	0.006	0.11	post
Glycerolipid metabolism	0.006	0.12	post
Leukocyte transendothelial migration	0.007	0.19	post
Hematopoietic cell lineage	0.009	0.17	post
Glutathione metabolism	0.012	0.14	post
Axon guidance	0.012	0.19	post
Galactose metabolism	0.013	0.15	post
Wnt signaling pathway	0.014	0.23	post
Type II diabetes mellitus	0.020	0.17	post
Fc epsilon RI signaling pathway	0.022	0.21	post
Adipocytokine signaling pathway	0.030	0.23	post
Glycolysis and gluconeogenesis	0.032	0.21	post
Apoptosis	0.041	0.22	post
Parkinsons disease	0.047	0.20	post
Regulation of autophagy	0.049	0.19	post
GenMAPP			
Cell cycle KEGG	<0.001	0.06	pre
DNA replication reactome	0.007	0.09	pre
Pyrimidine metabolism	0.008	0.08	pre
Apoptosis	<0.001	0.03	post
Integrin mediated cell adhesion KEGG	0.003	0.08	post
Apoptosis KEGG	0.003	0.09	post
Apoptosis GenMAPP	0.005	0.08	post
Nuclear receptors	0.007	0.07	post
Ubiquitin mediated proteolysis	0.012	0.14	post
GPCRDB class A rhodopsin like	0.013	0.23	post
Ovarian infertility genes	0.013	0.09	post
Galactose metabolism	0.017	0.07	post
Glutathione metabolism	0.017	0.10	post
Glycolysis	0.022	0.16	post
Gluconeogenesis	0.024	0.17	post
Striated muscle contraction	0.049	0.24	post

Table S6. Results of leading edge analysis using p<0.05 and FDR<0.25

Gene symbol	Protein	n gene sets
MNCs		
KEGG		
AKT1	PKB/Akt	15
MAPK3	ERK1	14
AKT2	RAC-beta	13
PRKCA	PKCalpha	13
PRKCB1	PKCbeta	12
RAC2	Rac2	12
PPP3CC	Calcineurin A-gamma	10
ITGB1	Integrin beta-1; CD29	9
PIK3R1	PI3K regulatory subunit 1	9
EGFR	EGFR	8
FYN	Proto-oncogene tyrosine kinase Fyn	8
ROCK1	ROCK	8
IKBKG	IKKgamma	8
NFKB1	NF-kB	8
Biocarta		
PRKCB1	PKCbeta	14
PRKCA	PKCalpha	14
MAPK3	ERK1	13
CALM3	calmodulin 3	11
CALM2	calmodulin 2	11
MAPK8	JNK	10
PPP3CC	Calcineurin A-gamma	10
MAPK1	ERK2	8
NFATC2	nuclear factor of activated t-cells	8
NFATC1	nuclear factor of activated t-cells	8
PRKAR1A	PKA type I-alpha	8
PIK3R1	PI3K regulatory subunit 1	7
GenMAPP		
ALDH1B1	Aldehyde dehydrogenase	6
ALDH9A1	Aldehyde dehydrogenase	6
ALDH1A1	Aldehyde dehydrogenase	6
ALDH1A2	Aldehyde dehydrogenase	6
ALDH1A3	Aldehyde dehydrogenase	6
LDHC	Lactate dehydrogenase	5
LDHB	Lactate dehydrogenase	5
EHHADH	Enoyl-CoA hydratase	4
ACAT2	Acetyl-CoA acetyltransferase	4
NFKB1	NF-kB	4
PDHB	Pyruvate dehydrogenase	4
PKM2	Pyruvate kinase isoenzymes M1/M2	4
PKLR	Pyruvate kinase isozymes R/L	4

Table S6. Continued

Gene symbol	Protein	n gene sets
CD34$^+$		
KEGG		
LCMT1	Leucine carboxyl methyltransferase	5
LCMT2	Leucine carboxyl methyltransferase	5
PRMT3	Protein arginine N-methyltransferase	5
PRMT6	Protein arginine N-methyltransferase	5
METTL2B	Methyltransferase-like protein 2B	4
PRMT2	Protein arginine N-methyltransferase	4
PRMT7	Protein arginine N-methyltransferase	4
PRMT5	Protein arginine N-methyltransferase	4
HEMK1	HemK Methyltransferase	4
CTH	cystathionase	4
CARM1	coactivator-associated arginine methyltransferase	4

Table S7. Cell counts and blood parameters of mice treated with simvastatin and/or welchol

	Control	Simvastatin	Welchol	Simvastatin + welchol
BM				
% LYMPH	58.3 ± 4.5	60.3 ± 5.0	66.7 ± 0.8*	63.2 ± 1.2†
% GRAN	13.4 ± 3.2	11.6 ± 3.3	9.2 ± 0.4	10.9 ± 0.6†
% MID	28.3 ± 2.1	28.1 ± 2.1	24.1 ± 0.5*	24.5 ± 2.8*‡
PB				
WBC (10^6/mL)	6.1 ± 2.1	7.0 ± 2.1	7.6 ± 1.5	7.3 ± 1.1
% LYMPH	86.8 ± 3.5	81.7 ± 7.2*	90.8 ± 0.9	85.3 ± 2.5
% GRAN	2.9 ± 1.5	3.2 ± 1.6	1.6 ± 0.6	2.7 ± 0.8
% MID	10.3 ± 2.6	15.1 ± 6.2*	7.6 ± 0.5	12.0 ± 1.7
HCT (L/L)	0.37 ± 0.03	0.37 ± 0.03	0.40 ± 0.01	0.39 ± 0.00
MCV (fl)	40.9 ± 1.7	41.0 ± 1.7	39.8 ± 0.1	39.4 ± 0.4
RBC (10^9/mL)	9.0 ± 0.7	9.0 ± 0.6	10.0 ± 0.2*	9.9 ± 0.2
HGB (mmol/L)	9.0 ± 0.7	9.1 ± 0.6	9.6 ± 0.2	9.3 ± 0.0
MCH (fmol)	1.0 ± 0.04	1.0 ± 0.04	1.0 ± 0.01	0.9 ± 0.02
MCHC (mmol/L)	24.5 ± 0.5	24.6 ± 0.5	24.1 ± 0.2	24.1 ± 0.2
RDW (%)	14.9 ± 0.6	14.8 ± 0.6	15.6 ± 0.3	15.7 ± 0.1
MPV (fl)	6.8 ± 0.2	6.9 ± 0.2	7.0 ± 0.1	7.2 ± 0.4*
PLT (10^6/mL)	540 ± 116	619 ± 109	605 ± 45	613 ± 279

BM: bone marrow; PB: peripheral blood; LYMPH: lymphocytes; GRAN: granulocytes; MID: other cells including monocytes, eosinophils, basophils and precursor white cells; WBC: white blood cells; HCT: hematocrit, MCV: mean corpuscular volume; RBC: red blood cells; HGB: hemoglobin; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red blood cell distribution width; MPV: mean platelet volume; PLT: platelets; *p<0.05 versus control; †versus welchol; ‡versus simvastatin.

7

Summary, general discussion & future perspectives

SUMMARY

Despite intensive treatment protocols and new developments in leukemia research, the long-term survival rates of adult AML patients are still low. For patients with a favorable prognosis, 5-year survival is about 50%, which is 4% in patients with an unfavorable prognosis, and these numbers are even lower for patients over 65 years. These low cure-rates are mainly due to the resistance of the patient's cancer cells to chemotherapy. As a consequence, there is a strong need for new therapeutic options to treat AML.

AML cells display an aberrant cholesterol homeostasis. In addition, these cells increase their cholesterol levels upon *in vitro* exposure to chemotherapeutic agents. As this acute cholesterol response is thought to protect against the cytotoxic effects of chemotherapy, blocking this response by cholesterol synthesis inhibitors could be able to sensitize the cells to cytotoxic agents. Therefore, a role for cholesterol synthesis inhibitors like statins has been put forward as a promising addition to standard anti-leukemic therapy in AML patients.

The aim of this thesis was to explore the potential benefits of the use of statins in treatment of AML. We focused on the use of statins in combination with other agents, as well as on the mechanism(s) behind (heterogeneity in) statin-induced effects.

In *chapter 2*, recent data on the role of cholesterol and the mevalonate pathway in AML are discussed. In addition, potential ways for intervention with the mevalonate pathway are described, among which is the application of the widely used plasma cholesterol-lowering statins.

In *chapter 3*, the potency of lovastatin to induce cytotoxic effects in AML cells was investigated in the absence and presence of commonly used chemotherapeutics. The majority of studies investigating cotreatment with statins make use of the total AML mononuclear cell fraction. However, we focused on the primitive AML $CD34^+$ cell fraction, which is enriched for leukemic stem cells, and compared this fraction with the more mature $CD34^-$ cells from the same patients. In both cell fractions, combining lovastatin and chemotherapy resulted in more pronounced cytotoxic effects, as determined by viability measurements and colony assays. Lovastatin alone was more effective in both normal and AML $CD34^+$ cells than in the AML $CD34^-$ cell fraction, indicating that primitive cells are more dependent on cholesterol synthesis for their survival. An interesting finding of this study was the difference in response within the AML $CD34^+$ patient samples to lovastatin; some AML samples responded similarly to normal $CD34^+$ cells, whereas other samples showed a reduced sensitivity to lovastatin. This distinction was, when subdividing $CD34^+$ cells into a primitive $CD34^+CD38^-$ fraction

and a more mature CD34$^+$CD38$^+$ fraction, most clearly seen in the CD34$^+$CD38$^-$ subfraction. These data imply that in a subset of AML patients especially the more primitive CD34$^+$ AML cell fraction displays a relative statin resistance. In the other, statin-sensitive patients chemotherapy was potentiated by lovastatin.

In *chapter 4* it was investigated whether simvastatin-induced effects could be potentiated by cotreatment with tipifarnib, an inhibitor of farnesylation. Tipifarnib is a farnesyltransferase inhibitor, which has been studied as a single agent in clinical trials in AML patients, resulting in variable outcomes. The beneficial effects of tipifarnib may be circumvented by alternative prenylation by geranylgeranyltransferase. However, simvastatin should, in theory, be able to block both geranylgeranylation and farnesylation. Therefore, combined treatment with simvastatin and tipifarnib was expected to result in additional effects. Again, CD34$^+$ AML cells were compared with the CD34$^-$ cell fraction. Both cell line data and data on patient AML cells showed that the combination of simvastatin and tipifarnib resulted in additive cytotoxicity, which coincided with a decrease in ERK phosphoryation. However, ERK phosphorylation was not decreased when either compound alone was used. In line with *chapter 3*, variability in responsiveness to simvastatin was mainly found within the AML CD34$^+$ subfraction. The AML samples could be divided in a normal and an abnormal responder group, and additive effects by cotreatment with tipifarnib only occurred in the normal responder group. From *chapter 3 and 4* we can therefore conclude that it is of importance to study the CD34$^+$ cell fraction, as the differences in response may have remained unnoticed when using the total mononuclear cell (MNC) fraction. In addition, almost all AML samples that were not sensitive to simvastatin were also not sensitive to tipifarnib, or the combination. These findings suggest a common mechanism of resistance, which, regarding the targets of both compounds, likely resides in the mevalonate pathway.

The aim of *chapter 5* was to obtain more insight in the mechanism behind the variability in responsiveness to statins among patient samples, as observed in *chapters 3 and 4*. To this end, cell lines with different sensitivities were tested, which all showed similar simvastatin-induced changes in cholesterol metabolism gene expression, indicating that simvastatin was functional in all cell lines. By discriminating between the cholesterol synthesis route and the isoprenylation route, using inhibitors and activators of either pathway, it appeared that simvastatin-induced cytotoxicity is not mediated by inhibition of cholesterol synthesis. In contrast, add-back experiments with intermediates of the isoprenylation route, i.e., farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), revealed that the isoprenylation pathway is responsible for simvastatin-induced cytotoxicity. Also in primary AML cells simvastatin-induced cytotoxic effects could be prevented by adding FPP or GGPP. Moreover, the isoprenylation pathway appeared to be responsible for the aforementioned differences in

sensitivity: inhibition of isoprenylation and ERK activity by simvastatin and other, more specific inhibitors was less efficient in insensitive cells. Together, our data indicate an important role for the isoprenylation route in the mechanism behind (heterogeneity in) statin-induced cytotoxicity.

The aim of *chapter 6* was to assess whether the *in vitro* observed effects of simvastatin, i.e., inhibition of cholesterol metabolism and isoprenylation, and chemosensitization, can also be detected in the *in vivo* situation. Besides using AML cell samples from patients that were treated with high-dose simvastatin, mice were treated with simvastatin as well. Upon treatment of AML patients with a high dosage simvastatin, no consequent changes in the expression of the cholesterol metabolism genes HMG-COAR, LDLR, ABCA1, and ABCG1 were observed in bone marrow MNCs of these patients, despite significant decreases in serum cholesterol levels in all patients. Still, in about 30% of the patients simvastatin treatment sensitized the AML $CD34^+$ cells to chemotherapy. The unexpected absence of changes in cholesterol metabolism gene expression was further investigated in mice. It appeared that, although liver cells displayed increased levels of HMG-COAR and LDLR mRNA after simvastatin treatment, murine bone marrow cells did not show increased levels of these genes. Results of gene set enrichment analysis on microarray data of AML patient cells revealed a set of pathways affected by simvastatin treatment that are dependent on isoprenylation. In combination with previous observations (*chapter 5*), this suggested that isoprenylation could be affected in bone marrow cells. Indeed, in mouse bone marrow cells, but not in liver cells, geranylgeranylation was inhibited, and in one of the four tested patient samples this inhibition was also detected. *In vitro* experiments showed that only inhibition of geranylgeranylation, but not farnesylation inhibition, takes place at physiological concentrations. In addition, this inhibition could solely be detected in bone marrow, but not in liver cells. The absence of inhibition of cholesterol synthesis and farnesylation is therefore likely dependent on the achieved simvastatin concentration. Overall, we showed that *in vivo* simvastatin concentrations are high enough to result in inhibition of geranylgeranylation, whereas inhibition of farnesylation and cholesterol synthesis remained unchanged in bone marrow MNCs.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Stem cell-enriched fraction versus total mononuclear cell fraction

Hematopoietic stem cells (HSCs) constitute the target for leukemic transformation for most AML subtypes[1]. Alternatively, more differentiated hematopoietic progenitors can acquire mutations that make these cells display stem-cell like properties[1]. These

leukemic stem cells possess unique properties that distinguish them from mature leukemic blasts. For example, the cells reside mostly in a quiescent cell cycle state and highly express a number of adenosine triphosphate (ATP)-binding cassette (ABC) transporters. ABC transporters transport a variety of substrates across extra- and intracellular membranes, and some of them are able to extrude chemotherapeutic agents from cells. As a consequence of the acquired chemoresistance, leukemic stem cells, in contrast to AML blasts, are not eradicated efficiently. Failure to adequately destroy this cell population often results in relapse of the disease.

Stem cell-enriched fractions of normal and leukemic hematopoietic cells display an increased expression of the cholesterol transporters ABCA1 and ABCG1[2,3]. In addition, the cholesterol synthesis and influx mediators HMG-COAR and LDLR are highly expressed in primitive hematopoietic cells[3]. This apparent increased cholesterol dependency suggests that these cells may be more sensitive to blocking of cholesterol synthesis by statins than their mature counterparts. In *chapter 3* we tested this hypothesis and showed that both normal and AML $CD34^+$ cells are more sensitive to lovastatin than $CD34^-$ cells. Another indication that (AML) $CD34^+$ cells respond differently to statins than more mature cells is presented in *chapter 6*. Upon treatment of patients with simvastatin, the pathways that were affected were different between $CD34^+$ and the $CD34^-$ and total MNC fraction. In the MNC and $CD34^-$ fraction pathways involved in cell signaling and immune responses were mainly affected, whereas the affected pathways in $CD34^+$ cells particularly involved protein synthesis. Interestingly, the cholesterol biosynthesis pathway was affected in $CD34^+$ cells, not $CD34^-$ cells, which again confirms the idea that $CD34^+$ may be more dependent on cholesterol synthesis than more mature cells. These data add to other reports that describe differences between $CD34^+$ and $CD34^-$ AML cells regarding long-term cell growth and susceptibility to apoptosis[4,5]. However, we have to increase our sample sizes and functionally test our findings further to be able to draw more solid conclusions.

Another interesting feature of $CD34^+$ cells is, as described in *chapter 3* and *4*, that differences in statin sensitivity are mainly found in this subfraction. Within the $CD34^+CD38^-$ fraction, which is even more enriched for stem cells[6], the difference in statin response among samples is most pronounced as compared with $CD34^+CD38^+$ cells. Therefore, it is useful to look at the most primitive subfraction of cells to predict which patients may benefit from statin treatment. Differences in response to simvastatin were also observed in total AML MNC samples in *chapter 5*, which could be a reflection of the high percentage of $CD34^+$ cells in some AML samples and the differences in response of these $CD34^+$ cells.

The downside of using $CD34^+$ cell fractions is the generally low number of $CD34^+$ cells present in the already limited amount of patient material, which limits the

number of experiments that can be done with a single AML sample. We have done microarray experiments to compare gene expression profiles of $CD34^+$ AML cells with those of $CD34^-$ cells. Comparable experiments with statin-treated AML cells could potentially provide more information on the mechanisms behind the difference in statin response between primitive and more mature AML subfractions. However, our experience is that it is not feasible to isolate sufficient numbers of $CD34^+$ cells from every patient sample. In addition, insight in cellular events that are triggered by statins in specific subfractions might be obtained in the future by phospho-protein FACS analysis. This novel method requires only low cell numbers and allows to assess the activity of proteins like ERK in $CD34^+$ cells compared with the $CD34^-$ fraction. Moreover, it will allow to distinguish between primitive cells and progenitor subfractions, such as common myeloid progenitors, granulocyte-macrophage progenitors, and megakaryocyte-erythroid progenitors. This method, set up for the measurement of phospho-STAT5[7], can be extended with other phospho-proteins. This type of experiments could provide answers to the questions why primitive hematopoietic cells are more sensitive to statins than more mature cells, and why heterogeneity in statin response resides in this primitive subfraction.

Lately, there has been a lot of discussion about the real identity of the leukemic stem cell. Although these cells were earlier characterized by the expression of CD34 and the lack of CD38 expression[8,9], there is now evidence that also among $CD34^+CD38^+$ cells and even $CD34^-$ cells leukemia-initiating capacity exists[10,11]. This previously unrecognized heterogeneity in leukemic stem cells makes it difficult to develop targeted treatment modalities.

Mechanism of action

Chapter 5 describes that simvastatin-mediated inhibition of the isoprenylation pathway, rather than of the cholesterol synthesis pathway, results in cytotoxic effects in AML cells. Isoprenoids GGPP and (partially) FPP were able to prevent simvastatin-induced cytotoxicity, whereas squalene, a precursor of cholesterol, was not. This is an interesting finding, since it was initially thought that statin-induced cytoxicity in AML was mediated by blocking cholesterol synthesis[12,13].

Research in multiple myeloma and lymphoma revealed a major role for protein geranylgeranylation, not farnesylation, in simvastatin-induced cytotoxicity[14,15]. This was based on the finding that, in contrast to FPP, GGPP rescued statin-induced effects, and that a geranylgeranyltransferase inhibitor (GGTI-298) was more effective than a farnesyltransferase inhibitor (FTI-277) in inducing cell death. We, however, found that the FTI tipifarnib was more effective in AML cells than GGTI-298. Moreover, the partial rescue by FPP can be explained by the fact that FPP is also used for the production of

squalene. Therefore, our data certainly do not exclude a role for inhibition of farnesylation by simvastatin.

The simvastatin-mediated block of the production of the isoprenoids FPP and GGPP results in decreased activation of proteins, like Ras and Rho, that require these isoprenoids for their proper function. This, in turn, leads to impaired cell signaling processes. We have shown that simvastatin inhibits phosphorylation of ERK, a downstream target of Ras, and that inhibition of Ras-signaling mimics statin-induced cytotoxic effects. In addition, constitutive activation of the Ras/MEK/ERK pathway has been shown to repress statin-induced apoptosis in AML cells[16]. Therefore, it is likely that this route plays an important role in statin-induced cytotoxicity. However, there are a number of additional proteins that require isoprenylation for their proper function[17]. This makes it difficult to pinpoint the exact route(s) via which statin-effects are exerted. Clearly, more research is required to obtain a more complete picture of the mechanisms involved. It is conceivable that, with new potential targets, more specific drugs can be designed to circumvent the side effects caused by high-dose statins.

Simvastatin exerts its various effects at different concentrations. For example, inhibition of geranylgeranylation generally occurs at concentrations that are 25-100 fold lower than the concentration required for inhibition of farnesylation and ERK-phosphorylation. Evidently, this has consequences for the *in vivo* situation, as the maximal dosage that can be safely given to patients results in plasma concentrations of up to 4 μM[18]. We were the first to investigate the molecular effects of *in vivo* applied simvastatin and found that in mice, and in a subset of patients, simvastatin treatment resulted particularly in inhibition of geranylgeranylation, and not of farnesylation. This finding indicates that for inhibition of geranylgeranylation sufficiently high plasma simvastatin levels are reached. Whether this inhibition results in biological effects remains to be elucidated, and should be addressed in further studies in AML mouse models and AML patients, as discussed below in more detail.

To gain more insight in the processes affected by *in vivo* simvastatin treatment, we performed a microarray analysis on AML cell samples from patients treated with simvastatin. This analysis revealed a number of pathways affected by the treatment, among which are many signal transduction routes that remain to be functionally confirmed. Cellular signaling pathways can be affected by disruption of lipid rafts. Lipid rafts are cholesterol-rich domains in cellular membranes, where mediators of signal transduction are concentrated. Inhibition of cholesterol synthesis by statins can result in disruption of lipid rafts[19]. However, our data indicated that a specific inhibitor of cholesterol synthesis, zaragozic acid A, does not induce cytotoxic effects in AML cell lines. This excludes the possibility that (only) inhibition of cholesterol synthesis, and the consequent disruption of lipid rafts, is responsible for cytotoxicity in AML cells.

Our *in vitro* studies were done in the presence of serum, a source of cholesterol. It is possible that the cholesterol-influx from the medium into the cell has overcome the initial cholesterol-mediated statin effects. However, experiments performed in low serum concentrations did not affect statin sensitivity[20].

Cotreatment with other agents

Although statins will not be used as single agent in AML treatment, these drugs are of potential interest in combination with other standard regimens or novel therapeutics. In this thesis, cotreatment of statins with the chemotherapeutics cytarabine, daunorubicin, and the clinically available farnesyltransferase inhibitor tipifarnib was investigated and indeed showed favorable additive effects.

In vitro chemotherapy results in increased cellular cholesterol levels in AML cells[13]. This acute cholesterol response can be blocked by statin treatment, and results in sensitization to chemotherapy in AML cells[12]. We showed that *in vitro* and *in vivo* sensitization to chemotherapy can be observed when using the $CD34^+$ subfraction that is enriched for leukemic stem cells. Clinical studies on the combinatory use of statins and chemotherapy are rare. The only study in AML patients, in which a high-dose statin was combined with chemotherapy, shows that this combination can safely be administered to patients. However, the study did not allow to draw valid conclusions about the antitumor efficacy of the combination treatment compared with single treatment due to the small sample size[21]. A drawback of statin treatment in combination with cytarabine is that statins induce cell cycle arrest at the G1 phase[22,23], and thereby possibly counteract the effects of cytarabine, which eradicates cells that are in S phase, resulting in less favorable clinical effects. The moment of administration of either compound may therefore influence the therapeutic outcome and should be studied further. The effects of daunorubicin are not dependent on cell cycle status, so in this case the moment of administration is not likely to influence the results.

Simvastatin and tipifarnib are compounds that (partially) interfere with the same pathway. Tipifarnib blocks farnesyltransferase, resulting in inhibition of farnesylation of proteins. In *chapter 5* it is shown that simvastatin induces cytotoxic effects not by inhibition of the cholesterol synthesis pathway, but by inhibition of the isoprenylation pathway, which can be both geranylgeranylation and farnesylation. Ras is a protein that can be both geranylgeranylated and farnesylated. Upon treatment with tipifarnib only, alternative prenylation by geranylgeranylation may bypass the inhibitory effects of tipifarnib on Ras signaling and consequently on cell survival[24], which is likely the cause of the disappointing clinical effects of tipifarnib monotreatment or combination treatment with chemotherapy[25-28]. As statins are capable of blocking both geranylgeranylation and farnesylation, it can be expected that the combined use of a statin and

tipifarnib will result in a more pronounced effect on cell survival. This was indeed the case, as illustrated in *chapter 4*, where additive cytotoxic effects were observed *in vitro* for the combination. In addition, phosphorylation of ERK, a downstream target of Ras, was inhibited by the combined use of simvastatin and tipifarnib, whereas the individual compounds at the same concentrations did not affect ERK phosphorylation. Thus, in AMLs that display increased activation of ERK, e.g., due to constitutively active Ras, combined treatment with simvastatin and tipifarnib may be more effective in inducing cytotoxic effects. Achieved plasma concentrations of tipifarnib are sufficiently high to inhibit farnesylation (0.5-1 μM)[29]. In addition, we have shown that in a subset of patients high-dose statin treatment resulted in inhibition of geranylgeranylation in bone marrow cells. Thus, combined treatment with statins and tipifarnib is likely to be beneficial in a subset of AML patients and should be investigated *in vivo*.

To bypass alternative prenylation, combination treatment with a farnesyltransferase inhibitor and a geranylgeranyltransferase inhibitor (GGTI) may be even more effective[30], but currently there are no clinically applicable GGTIs. In addition, although FTIs and GGTIs are indeed reported to act synergistically[30], the combined treatment would clinically not be feasible due to high toxicity[31].

Heterogeneity in response

Among patient AML samples there are marked differences in response to *in vitro* statin treatment. From a clinical point of view, it is of importance to know which patient will respond to statin treatment, as this would allow to predict which patient would benefit from treatment. We tried to find the rationale behind this heterogeneity in response by using AML cell lines with differences in susceptibility to simvastatin. *Chapter 5* shows that AML cell lines with a decreased susceptibility to simvastatin show less efficient inhibition of isoprenylation by simvastatin, lower basal phosphorylated ERK levels, and that simvastatin-induced cytotoxicity can less efficiently be prevented by intermediates of the isoprenylation pathway. Moreover, isoprenylation inhibitors, as well as a MEK inhibitor, were less effective in statin-insensitive cell lines. These findings demonstrate a role for the Ras/MEK/ERK signal transduction pathway in statin resistance. This is an interesting observation, since activating *RAS*-mutations are found in about 30% of the AML patients, and autocrine production of growth factors and cytokines by AML cells or mutations in their receptors can activate the Ras/MEK/ERK survival route as well[32,33]. However, from our cell line data and data from others[34,35] can be concluded that the Ras status of the AML cells can be dissociated from their response to simvastatin. Still, it appears that sensitive cell lines are more dependent on the Ras/MEK/ERK route for their survival than insensitive cell lines.

In insensitive cell lines, a ~10-fold higher simvastatin concentration is required to obtain similar effects to those in sensitive cell lines. However, only a 2-fold higher concentration of the MEK inhibitor is required for comparable cytotoxic effects in the insensitive cell lines. Thus, the heterogeneity in sensitivity to statins cannot be explained solely by differences in the MEK/ERK pathway. However, inhibitors of the Rho-ROCK pathway and the PI3K/Akt pathway were equally effective in the sensitive and insensitive cell lines, indicating that these routes do not account for the heterogeneous response to simvastatin. The isoprenylation route needs to be further dissected to elucidate more routes involved in simvastatin resistance. Microarray analysis on a high number of sensitive and insensitive cell lines will aid us in finding these differentially regulated pathways.

Whether the isoprenylation pathway is also the key to differences in statin sensitivity among primary AMLs remains to be investigated. Intervention with the isoprenylation pathway was not equally effective among the AML samples, but this heterogeneity in isoprenylation inhibition did not correlate with statin responsiveness. However, we should realize that the absence of this correlation may be due to the cells used: in these experiments the total MNC fraction, not the $CD34^+$ fraction, was used, and differences in statin sensitivity were mainly observed in the $CD34^+$ fraction. It can be expected that for patient AML samples, and ultimately for patients in the clinic, that the mechanisms behind heterogeneity in response to statins are more complicated than can be predicted on the basis of cell line studies. Many different AML subtypes exist, with for example different differentiation statuses, cytogenetics, or additional mutations, which all contribute to the phenotype of these cells. This phenotype affects sensitivity to therapy, like observed with all-trans retinoic acid (ATRA) treatment in patients with promyelocytic leukemia (M3) and tyrosine kinase inhibitors in patients with *c-Kit* mutations, and this is likely to hold true for statin treatment as well. In patients, the mechanism behind simvastatin response may be even more complicated by different activities of CYP3A4 among patients, the enzyme that converts statins, such as lovastatin and simvastatin, into their active form[36].

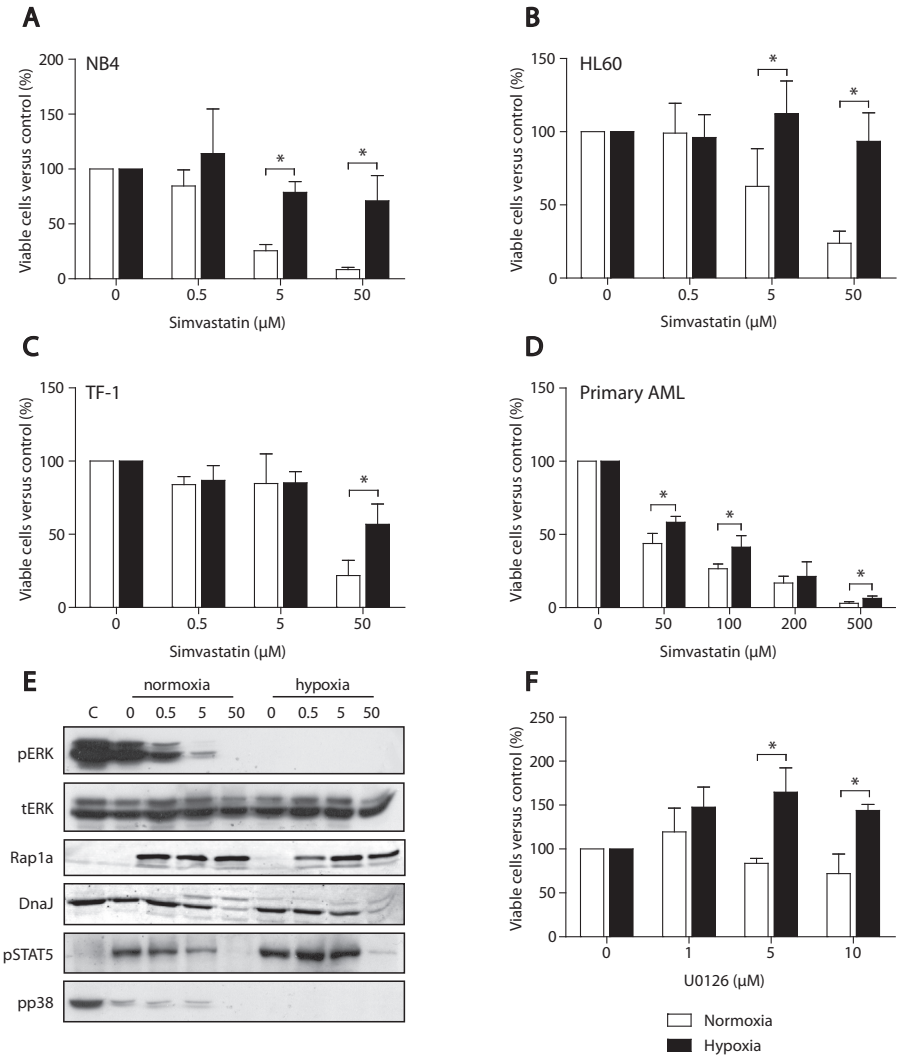
An important follow-up project would be to extend the simvastatin clinical trial and define patients with and without biological and clinical response to (co-)treatment with simvastatin. Microarray analysis on material of these AML patients could provide us with genes and pathways that are differentially expressed or regulated between the normal and abnormal simvastatin responders. By pinpointing biomarkers, it should in the future be possible to predict which patients may benefit from statin treatment.

Microenvironment

The *in vitro* experiments shown in this thesis have been done in culture medium. A disadvantage of this approach is that the cells are studied outside their natural environment, called the microenvironment, or niche. Increasingly, the microenvironment is considered to play an important role in normal hematopoiesis and leukemogenesis[37]. The microenvironment can interact with AML cells and produce cytokines for growth and survival[38]. One way in which this microenvironment can be mimicked is by using a stromal cell line[4]. The bone marrow stroma is known to consist of several cell types, including fibroblasts, adipocytes, osteoblasts, and endothelial cells. There is evidence that these stromal cells can protect leukemia cells against the cytotoxic effects of chemotherapy[39]. In addition, statins affect growth and differentiation of stromal cells at clinically relevant concentrations[40,41]. Future experiments should therefore be done to assess what the influence of stromal cells is on statin sensitivity of leukemic cells, and to determine the effects of statins on these cells and the consequences for leukemic cell survival.

Another feature of the bone marrow microenvironment is the oxygen level, which is much lower (about 2%) in the bone marrow niche as compared to oxygen levels in the peripheral blood (21%)[42]. This low oxygen level has a significant impact on the metabolic state of the cell and on the amount of available energy in the form of ATP. Therefore, we initiated experiments where sensitivity to simvastatin was assessed under low and high oxygen conditions.

In human AML cell lines, we tested simvastatin sensitivity by assessing cell counts after 24 pre-incubation under normoxic (21% O_2) or hypoxic (2% O_2) conditions, followed by 48 hours of simvastatin treatment. Especially in the simvastatin-sensitive cell lines, but also in TF-1 cells, simvastatin was less effective in lowering cell numbers under hypoxic conditions (Figure 1A). This was also found in primary human AML MNCs. Inhibition of both farnesylation and geranylgeranylation by simvastatin appeared to be not notably affected by hypoxia, and thus appeared not responsible for the diminished statin sensitivity. More striking was the finding that GM-CSF-induced phosphorylated ERK was (almost) completely downregulated under hypoxia (Figure 1C). This is in contrast to other findings, where upregulation of phospho-ERK was observed in AML cells, albeit under higher O_2 levels (6%), and without stimulation by cytokines[43], but appears to be in line with the findings under hyperoxia[44]. In this latter study, increased phospho-ERK levels were observed under high (95%) oxygen compared with normoxic conditions. In addition, we showed that U0126, a MEK inhibitor, was less effective under hypoxia, and even seemed to increase cell proliferation when applied



under hypoxic conditions (Figure 1D and data not shown). The decreased activation of ERK was not GM-CSF receptor-dependent, since no difference in STAT5 phosphorylation was observed under both conditions (Figure 1C and data not shown).

Alternatively, downregulation of phospho-ERK under hypoxic conditions may be linked to metabolic changes. Cells under hypoxic conditions switch their metabolism towards anaerobic glycolysis, thereby generating less energy, which is accompanied many cellular changes in order to facilitate an adaptation to the hypoxic conditions. Hypoxia-inducible factors mediate a number of these cellular responses to hypoxia

◂ *Figure 1. Decreased cytotoxicity by simvastatin in AML cells under hypoxia is MEK/ERK dependent.* (A) NB4, (B) HL60 and (C) TF-1 cells were cultured under normoxic (21% O_2) or hypoxic (2% O_2) conditions for 24 hours, replated under the same conditions, and treated for 48 hours with different concentrations of simvastatin. Cells were counted and dead cells were excluded by trypan blue staining, which remained constant upon treatment. Data are presented as mean ± SD (n=4). (D) Primary human AML cells (n=3) were cultured under normoxic or hypoxic conditions for 24 hours, and treated for 24 hours with different concentrations of simvastatin, after which cell counts were determined as described above. Data are presented as mean ± SD. (E) After culturing and treatment as described, cell lines were stimulated for 15 minutes with GM-CSF prior to harvesting. Western blot was done for phospho-ERK, total ERK, which served as a loading control, phospo-STAT5, phospho-p38, Rap1a, a protein that is exclusively geranylgeranylated, and DnaJ, a protein that is exclusively farnesylated. The Rap1a blot shows the unprenylated protein; of DnaJ the upper band represents the unprenylated protein, the lower band is the prenylated protein. Experiments were done at least 3 times and a representative example of each protein blot for HL60 cells is shown; comparable results were obtained in NB4 and TF-1 cells. (F) Cells were treated with MEK inhibitor U0126 and cell counts were done. Data are shown as mean ± SD (n=3) for NB4; comparable results were obtained with HL60 cells. Student's *t*-test was done to calculate significance levels.

by regulating gene expression. However, $CoCl_2$ treatment, which stabilizes hypoxia-inducible factor 1α (HIF1α) and thus mimics hypoxia-induced HIFα activity, did not change phospho-ERK levels (data not shown).

AMP-activated protein kinase (AMPK) acts as an energy sensor and regulates a number of metabolic processes, including fatty acid metabolism and sterol synthesis[45]. Under hypoxic conditions, AMP-activated protein kinase (AMPK) is upregulated[45]. It has been reported that HMG-CoAR is downregulated by AMPK[45]. This potentially explains why cells under hypoxic conditions are less sensitive to simvastatin treatment, as HMG-CoAR is the direct target of statins. However, our preliminary data do not show changes in HMG-CoAR gene expression under hypoxia, and therefore do not support this hypothesis.

An explanation for the absence of active ERK may be that the decreased cholesterol synthesis under hypoxia results in a decreased lipid raft formation. In contrast to ERK, STAT5 is not lipid raft-dependent[46], which explains why STAT5 is still phosphorylated by GM-CSF stimulation under hypoxia. Other insights come from data on macrophages cultured under hyperoxic conditions, i.e., 95% O_2, that show an increase of pERK compared to normoxia[44]. This increase was due to a decreased ERK-directed phosphatase activity. Preliminary microarray data generated by our lab revealed increased expression of a subset of phosphatases in cord blood $CD34^+$ cells grown under hypoxia. This may well correlate with increased phosphatase activity, which provides another possible explanation for the hypoxia-mediated decrease in ERK phosphorylation. We are currently investigating the stated hypotheses concerning the inhibition of phospho-ERK.

Together, our preliminary data show that AML cells are less sensitive to statin treatment under hypoxic conditions that resemble their natural environment. This decreased sensitivity may be, at least in part, due to decreased activity of ERK. The exact mechanism behind this diminished ERK activity and the alternative pathways that take care of survival of these cells under influence of hypoxia remain to be determined. It also unclear how these findings can be translated to patients and what the consequences may be for clinical treatment. As hypoxic conditions, which are present in the bone marrow, decrease sensitivity of AML cells to simvastatin, the administered simvastatin dosage should be even higher than the one derived from previous *in vitro* data.

The use of a NOD-SCID mouse model would provide us with the natural bone marrow microenvironment. The only report on statin use in a SCID mouse model describes that, upon injection of HL60 cells, simvastatin reduced the number of these cells[35]. However, as we show in this thesis, these cells are already sensitive to a low dose simvastatin, in contrast to primary AMLs, making it a less suitable model. To obtain a more reliable idea of the situation in patients, patient AML cells should be transplanted into NOD-SCID mice[47]. The disadvantage of this approach is that the heterogeneity between patients makes it difficult to interpret the results obtained, and not all AML samples are able to engraft in mice[48]. Moreover, as the Ras/MEK/ERK route is important for cell survival, and as this pathway is deactivated under hypoxia, there are likely other survival pathways active that are not, or less affected by simvastatin.

Role of simvastatin concentrations

In vitro findings do not always reflect the *in vivo* situation, which may be caused by the influence of the microenvironment on the tested cells, as described above. In addition, pharmacokinetic aspects that apply when statins are administered to patients are also likely to play a role. Although *in vitro* experiments revealed simvastatin-induced changes of the expression of cholesterol metabolism genes, similar changes were not observed in MNC cells of simvastatin-treated AML patients. However, simvastatin treatment did exert its actions, as it reduced serum cholesterol levels in the patients. In addition, inhibition of isoprenylation could be observed in MNCs of mice and a subset of patients treated with simvastatin. Inhibition of isoprenylation occurred *in vitro* at low concentrations, whereas changes in cholesterol metabolism gene expression were noted at higher, physiologically irrelevant concentrations. Therefore, it is evident that certain biological effects of statins are dependent on the plasma concentration that is achieved.

There are a number of factors that influence the physiologically achieved concentration of statins. For example, statins undergo an extensive first pass metabolism by the liver when orally applied[36]. This results in a low bioavailability, and, as a consequence,

the plasma concentrations are much lower than one would expect based on the dosage applied. With an applied dosage of 4 mg/kg, maximum lovastatin plasma levels of 0.1-3.9 μM could be achieved in cancer patients[18]. A way to circumvent the first pass effect is by administering the drug intravenously. Unfortunately, intravenously applicable formulations of statins are not available[49].

Both lovastatin and simvastatin are substrates for CYP3A4, which has, as already discussed, a variable activity in humans[36]. This can be caused by genetic differences and extrinsic factors, such as food and drug intake, which makes it difficult to predict the actual concentration that will be achieved in the patient. Inhibition of CYP3A4 may result in higher statin plasma levels[50], although intraindividual differences will still be apparent. Furthermore, this approach increases the risk on side effects, and may only be feasible when the patient is extensively monitored. Besides CYP3A4, there are several other gene products that vary among patients, and that contribute to the *in vivo* pharmacokinetics of statins[51].

Another disadvantage of statins like simvastatin and lovastatin is their short half-life, resulting in peak-concentrations that last for only a few hours[36]. This is in contrast with the situation during *in vitro* experiments, in which statin concentrations remain high throughout the experiment. As a consequence, the drug has to be administered frequently as to achieve continuous maximum drug levels. Synthetic statins are available with a longer half-life (rosuvastatin, atorvastatin), but these statins are reported to be less effective in AML cells *in vitro* than the natural compounds[52]. A clinical trial with pravastatin, a natural statin with a relatively long half-life, showed encouraging clinical responses in AML patients[21]. For example, the complete remission rate of newly diagnosed patients with unfavorable cytogenetics and first relapse patients was higher than the historical complete remission rates. However, the small number of enrolled patients as well as the retrospective comparison do not allow to conclude whether the treatment was beneficial.

Increasing the statin dosage to achieve higher plasma levels will likely result in more side effects. We and others have shown preclinically that treatment with statins in combination with other agents such as chemotherapeutics and farnesyltransferase inhibitors can have additive cytotoxic effects in AML cells. This suggests that the use of lower, nontoxic dosages of both compounds may be an interesting approach for the treatment of AML patients.

Conclusion

Interference with the mevalonate pathway with statins is a potentially interesting therapeutic direction for the treatment of AML. We have shown by *in vitro* experiments that statins are effective in inducing cytotoxic effects by blocking isoprenoid synthesis and

the subsequent signal transduction routes, but not by inhibiting cholesterol synthesis. However, *in vivo* effects are less pronounced, probably due to limited plasma statin concentrations reached. Statins exert most of their effects on AML cells at concentrations that cannot be achieved in patients, without inducing severe side effects. Also the influence of the microenvironment should be taken into account: preliminary data revealed that simvastatin is less effective under hypoxic conditions. Interestingly, inhibition of geranylgeranylation occurs at low concentrations *in vitro* and in the *in vivo* situation. We showed that one way to increase the efficacy of statins may be to combine statin treatment with other agents like farnesyltransferase inhibitors. The potential of this combination treatment should be investigated further by doing *in vivo* studies. Future research is warranted to further improve our knowledge on especially the *in vivo* effects on statins, taking into account the role of the microenvironment, and pinpointing the cellular effects that occur at low concentrations in AML cells. In addition, patient samples differ in their response to statins, especially within the more primitive $CD34^+$ cell fraction. This heterogeneity in response is present in patients as well. We have made a start in dissecting the mechanism behind the heterogeneity in cellular responses. However, more research is required to reveal the exact mechanism(s) of action, which can hopefully lead to the identification of predictive biomarkers for statin treatment effects in AML patients.

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8

Nederlandse samenvatting

NEDERLANDSE SAMENVATTING

Hematopoiese, acute myeloide leukemie en de (leukemische) stamcel

Hematopoiese omvat de vorming van de verschillende bloedceltypen tijdens het leven van een organisme. Dit proces begint bij de onrijpe hematopoietische stamcel (HSC), die zichzelf vermenigvuldigt en daarnaast dochterbloedcellen produceert. Deze dochtercellen ontwikkelen zich vervolgens in een aantal stappen tot volgroeide bloedcellen, waaronder rode en witte bloedcellen. Het proces van hematopoiese is dus volgens een hiërarchische structuur opgebouwd. De processen van vermenigvuldiging en ontwikkeling (differentiatie) van de bloedcellen worden zorgvuldig gereguleerd. Bij patiënten met acute myeloide leukemie (AML) zijn deze processen ontregeld. Daardoor differentiëren de cellen zich niet meer, maar vermenigvuldigen zich nog wel. Dit resulteert in een ophoping van onrijpe bloedcellen (blasten) in het beenmerg, en op termijn ook in het bloed. AML wordt behandeld met chemotherapie en in bepaalde gevallen wordt de patiënt getransplanteerd met beenmergstamcellen van een gezonde donor. Ondanks het feit dat er de afgelopen jaren veel vooruitgang is geboekt in de behandeling van AML patiënten, zijn de vooruitzichten voor deze patiënten op de langere termijn zeer matig. Daarom is het van belang onderzoek te verrichten naar andere vormen van therapie.

De laatste jaren is de aandacht verschoven van het uitroeien van de opgehoopte leukemische blasten naar het uitschakelen van de leukemische stamcellen (LSCs). Net als de HSCs zorgen de LSCs voor bloedcelvorming, maar dan voor de leukemische cellen in plaats van de normale bloedcellen. Behandeling met chemotherapeutica doodt weliswaar de meeste leukemische cellen, maar het is inmiddels bekend dat chemotherapie waarschijnlijk niet in staat is de LSCs volledig uit te roeien, wat tot gevolg heeft dat de ziekte uiteindelijk vaak terugkomt. Daarom is het van belang op zoek te gaan naar een behandeling die de LSCs wel volledig uitroeit. Zowel de HSCs als de LSCs dragen een bepaald eiwit op hun oppervlak. Dit eiwit, CD34, stelt ons in staat om de stamcellen van de volwassen cellen te scheiden en zo te onderzoeken of een behandeling effectief is voor deze specifieke subpopulatie.

Het cholesterol metabolisme in acute myeloide leukemie

Het is bekend dat er een relatie is tussen AML en een verstoord cholesterol metabolisme. Cholesterol is een vetachtige stof die nodig is voor de opbouw van de celmembranen. Daarin zorgt cholesterol voor de 'stevigheid' van de celmembraan en voor het functioneren van signaaleiwitten die aan de membraan verbonden zijn. De hoeveelheid cholesterol binnen een cel wordt nauwkeurig gereguleerd door middel van gecontroleerde opname, afgifte en productie. Cholesterol wordt gemaakt in een aantal stappen welke onderdeel zijn van de mevalonaatroute. Het enzym HMG-CoA reductase

(HMG-CoAR) speelt hierin een belangrijke rol. Opname van cholesterol verloopt via de LDL receptor (LDLR) en afgifte van cholesterol gaat via transporters die ABCA1 en ABCG1 worden genoemd.

In AML-cellen is aangetoond dat de genexpressie en activiteit van HMG-CoAR en LDLR hoger is dan in normale beenmergcellen. Dit is hangt samen met een langere overleving van de cellen. Daarnaast laten deze cellen een acute verhoging van hun cholesterolspiegels zien zodra ze *in vitro* (= in kweek) worden blootgesteld aan chemotherapie. Men denkt dat deze acute cholesterol respons de cellen beschermt tegen de toxische effecten van chemotherapeutica. Deze bevindingen hebben geleid tot de hypothese dat cholesterolverlagers, zoals statines, een effectieve toevoeging kunnen zijn op de huidige antileukemische therapieën.

Doel van het onderzoek

Het doel van dit proefschrift was om het effect van statines op AML-(stam)cellen vast te stellen, waarbij is gefocust op het gebruik van statines in combinatie met andere geneesmiddelen en op het achterliggend mechanisme van de gevonden effecten.

Samenvatting

In *hoofdstuk 2* wordt een up-to-date overzicht gegeven van de rol van cholesterol en de mevalonaatroute in AML. Ook worden mogelijke manieren van interventie met deze route beschreven, waaronder de algemeen gebruikte cholesterolverlagende statines.

In *hoofdstuk 3* is gekeken of de cholesterolverlager lovastatine in staat was om het effect van veelgebruikte chemotherapeutica zoals cytarabine en daunorubicine te versterken. Hiervoor zijn uit het beenmerg van patiënten primitieve, CD34-positieve AML cellen (de zogenaamde $CD34^+$ fractie) geïsoleerd en bestudeerd en deze fractie hebben we vergeleken met de rijpere $CD34^-$ fractie van dezelfde patiënten. In beide fracties resulteerde de combinatiebehandeling met lovastatine en chemotherapie in meer uitgesproken celdodende effecten dan met lovastatine of chemotherapie alleen. Wanneer de cellen alleen met lovastatine werden behandeld bleken de normale en de $CD34^+$ AML cellen gevoeliger te zijn dan de $CD34^-$ AML cellen. Dit laat zien dat primitieve (stam)cellen voor hun overleving afhankelijker zijn van cholesterolsynthese dan de rijpere cellen. Een andere bevinding van deze studie was het verschil in gevoeligheid tussen de $CD34^+$ AML patiëntenmonsters: sommige AML-monsters waren gevoelig voor lovastatine, terwijl andere monsters een (sterk) verminderde gevoeligheid voor lovastatine lieten zien. Deze bevindingen impliceren dat in een deel van de AML-patiënten met name de primitievere $CD34^+$ AML celfractie een relatieve statine-resistentie vertoont. In de andere, gevoelige patiëntenmonsters werd het effect van chemotherapie versterkt door lovastatine.

Statines remmen de eerder genoemde mevalonaatroute, wat niet alleen resulteert in een afname van de productie van cholesterol, maar ook in verminderde productie van bijproducten van deze route, zoals de isoprenoïden farnesylpyrofosfaat (FPP) en geranylgeranylpyrofosfaat (GGPP). Deze isoprenoïden kunnen binden aan bepaalde eiwitten, een proces dat isoprenylatie heet. Deze binding is nodig voor de functie van de eiwitten, opdat ze signalen kunnen afgeven aan de cel en zijn omgeving om bijvoorbeeld celvermenigvuldiging of bescherming tegen celdood te bewerkstelligen. Een geneesmiddel dat op een andere wijze dan statines interfereert met isoprenoïden is tipifarnib. Dit geneesmiddel voorkomt de binding van FPP aan de signaaleiwitten. In *hoofdstuk 4* is onderzocht of de effecten van de cholesterolverlager simvastatine konden worden versterkt door mede te behandelen met tipifarnib. Ook hier werden $CD34^+$ AML cellen vergeleken met $CD34^-$ cellen. Zowel experimenten met gekweekte cellen (cellijnen) als met cellen van AML patiënten lieten zien dat het combineren van simvastatine met tipifarnib de celdodende effecten versterkte, met name in de AML $CD34^+$ celfractie. Dit viel samen met een afname van de actieve vorm van het signaleringseiwit ERK door de combinatiebehandeling. Dit verschijnsel trad echter niet op wanneer dezelfde concentratie simvastatine of tipifarnib apart werden gebruikt. Ook in deze experimenten werd een verschil in gevoeligheid binnen de $CD34^+$ AML fractie gevonden. Er kon onderscheid worden gemaakt tussen AML-monsters met een 'goede' en een 'slechte' respons, waarbij de versterkte effecten door combinatiebehandeling alleen zichtbaar waren in de goede respons-groep. Bijna alle AML-monsters die ongevoelig waren voor simvastatine waren ook ongevoelig voor tipifarnib of de combinatie ervan. Dit suggereert dat er een gemeenschappelijk resistentiemechanisme is, die, gezien de aangrijpingspunten van beide stoffen, zeer waarschijnlijk in de mevalonaatroute gelokaliseerd is. De resultaten van *hoofdstuk 3 en 4* geven aan dat het van belang is om de $CD34^+$ celfractie binnen de totale fractie te bestuderen, aangezien verschillen in gevoeligheid waarschijnlijk onopgemerkt blijven als de totale (mononucleaire) celfractie wordt gebruikt.

Het onderzoek beschreven in *hoofdstuk 5* had als doel meer inzicht te verkrijgen in het mechanisme achter de verschillen in gevoeligheid van patiëntenmateriaal voor statines die we hebben waargenomen in *hoofdstuk 3* en *4*. Hierbij is gebruik gemaakt van verschillende cellijnen die allemaal een andere gevoeligheid voor simvastatine vertonen. Uit deze onderzoekingen is naar voren gekomen dat de celdodende effecten van simvastatine niet tot stand komen door remming van de cholesterolsynthese, maar door remming van de isoprenylatieroute. Ook in AML cellen van patiënten kon dit mechanisme van simvastatine worden aangetoond. Bovendien laten we zien dat de isoprenylatieroute verantwoordelijk is voor het optreden van de hierboven vermelde verschillen in gevoeligheid voor simvastatine. Samengevat laten deze gegevens zien

dat de isoprenylatieroute een belangrijke rol speelt in de statine-geïnduceerde celdood en de variatie in statine-geïnduceerde celdood.

In *hoofdstuk 6* is onderzocht of de effecten van simvastatine die *in vitro* zijn waargenomen, namelijk de remming van het cholesterolmetabolisme en isoprenylatie en het gevoeliger maken van cellen voor chemotherapie, ook worden waargenomen in de *in vivo* (= in leven, in dieren en mensen) situatie. Hiervoor zijn AML patiënten behandeld met een hoge dosering simvastatine. Bij de start en het staken van de simvastatine-behandeling zijn AML-monsters genomen welke we hebben onderzocht. Op vergelijkbare wijze zijn ook muizen behandeld en onderzocht. De resultaten toonden aan dat behandeling van AML patiënten met een hoge dosis simvastatine resulteerde in inconsistente veranderingen in expressie van cholesterolmetabolisme-genen in beenmergcellen, ondanks een sterke afname in de serum cholesterolspiegels in alle patiënten. Toch vonden we in 30% van de patiënten dat behandeling met simvastatine de AML $CD34^+$ cellen gevoeliger maakte voor chemotherapie. Het onverwacht uitblijven van veranderingen in cholesterolmetabolisme-genen is verder onderzocht in muizen. Omdat statines na inname als eerste de lever bereiken, verwachtten we dat cholesterolmetabolisme-genen in levercellen veranderd tot expressie zouden komen. In de levers van muizen bleek dit inderdaad het geval, echter, de genen bleven onveranderd in beenmergcellen van de muizen na behandeling met simvastatine. Op patiëntenmateriaal dat was verkregen voor en na behandeling met simvastatine is vervolgens een microarray analyse gedaan. Hiermee kan de expressie van praktisch alle genen in een cel vastgesteld worden. Met deze microarraygegevens is een 'gene set enrichment analysis' uitgevoerd, een methode waarmee aangetoond kan worden welke signaleringsroutes verrijkt zijn, bijvoorbeeld na behandeling met simvastatine. Deze analyse bracht een aantal routes aan het licht die afhankelijk zijn van isoprenylatie. In combinatie met eerdere bevindingen *(hoofdstuk 5)* suggereerde dit dat isoprenylatie werd beïnvloed in beenmergcellen. Inderdaad bleek dat in beenmergcellen van muizen geranylgeranylatie, een vorm van isoprenylatie, geremd was. Ook in een deel van de geteste patiëntenmonsters werd remming van geranylgeranylatie aangetoond. Remming van farnesylatie, de andere vorm van isoprenylatie, was niet zichtbaar in beenmerg van muizen noch patiënten. *In vitro* experimenten lieten zien dat alleen remming van geranylgeranylatie in beenmergcellen plaatsvond bij concentraties die in de patiënt bereikt kunnen worden. De afwezigheid van remming van cholesterolsynthese en farnesylatie in het beenmerg is daarom wellicht te verklaren door een te lage simvastatine concentratie in het bloed. Samengevat lieten deze studies zien dat *in vivo* bereikte simvastatineconcentraties hoog genoeg zijn om geranylgeranylatie in beenmergcellen te remmen, terwijl remming van farnesylatie en cholesterolsynthese bij deze concentratie niet optreedt.

Conclusies van het onderzoek

Blokkering van de mevalonaatroute met statines is een potentieel interessante therapeutische benadering voor de behandeling van AML. In dit proefschrift is aangetoond dat de gevoeligheid voor statinebehandeling erg varieert tussen patiënten(monsters). Omdat het van belang is te kunnen voorspellen welke patiënten baat zullen hebben bij een bepaalde behandeling, in dit geval statines, is het belangrijk om te weten waardoor dergelijke verschillen in gevoeligheid optreden. Dit onderzoek heeft aangetoond dat deze verschillen (voor een groot deel) in de isoprenylatieroute liggen en dat deze route ook verantwoordelijk is voor de celdodende effecten van statines. Een probleem is dat deze effecten niet allemaal te bewerkstelligen waren met een hoge dosis simvastatine in patiënten. Echter, bij deze niet-toxische dosis vindt wel remming van geranylgeranylatie plaats in een deel van de patiënten. Door statines te combineren met andere geneesmiddelen zou deze remming verder kunnen worden benut. We hebben *in vitro* aangetoond dat chemotherapie en tipifarnib de effecten van statines kunnen versterken, ook in de meer primitieve cellen, maar de geschiktheid van dergelijke combinatiebehandelingen zal verder onderzocht moeten worden in *in vivo* studies. Ook zal meer onderzoek verricht moeten worden om nauwkeuriger de mechanismen achter de verschillen in gevoeligheid voor statines te onthullen. Hierdoor kunnen op den duur biomarkers geïdentificeerd worden aan de hand waarvan men kan voorspellen welke AML-patiënten baat zullen hebben bij behandeling met statines.

Appendix

Dankwoord
Abbreviations
Publications

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&

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Abbreviations

ABC	ATP-binding cassette
ABCA1	ABC transporter A1
Acetyl-CoA	Acetyl Co-enzyme A
ALL	Acute lymphoid leukemia
AML	Acute myeloid leukemia
AMPK	AMP-activated protein kinase
ApoA-1	Apolipoprotein A-1
ApoE	Apolipoprotein E
ARA-C	Cytarabine
ATP	Adenosine triphosphate
ATRA	All-trans retinoic acid
BM	Bone marrow
BP	Bisphosphonate
CD	Cluster of differentiation
CDC42	Cell division control protein 42 homolog
cDNA	Complementary DNA
CENP	Centromere protein
CFC	Colony-forming cell
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CR	Complete remission
CYP3A4	Cytochrome P450 3A4
dKO	Double knockout
DNA	Deoxyribonucleic acid
DNR	Daunorubicin
EFS	Event free survival
EPO	Erythropoietin
ERK	Extracellular signal-regulated kinase
FAB	French-American-British
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FDR	False discovery rate
FLT-3	Fms-related tyrosine kinase-3
FPP	Farnesyl pyrophosphate
FTase	Farnesyltransferase
FTI	Farnesyltransferase inhibitor

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GDP	Guanosine diphosphate
GMP	Granulocyte/macrophage progenitor
GGPP	Geranylgeranyl pyrophosphate
GGTase	Geranylgeranyltransferase
GGTI	Geranylgeranyltransferase inhibitor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GSEA	Gene set enrichment analysis
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
GVHD	Graft versus host disease
HC	Hypoxycholesterol
HDL	High-density lipoprotein
HIF	Hypoxia-inducible factor
HMG-COA	3-hydroxy-3-methyl-glutaryl-COA
HMG-COAR	3-hydroxy-3-methyl-glutaryl-COA reductase
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
IL-3	Interleukin-3
KO	Knockout
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LFA-1	Leukocyte function-associated antigen 1
LSC	Leukemic stem cell
LSK	Lin^- $sca\text{-}1^+$ $c\text{-}Kit^+$cells
LT-HSC	Long-term hematopoietic stem cell
LXR	Liver X receptor
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
MEP	Megakaryocyte/erythroid progenitor
MM	Multiple myeloma
MNC	Mononuclear cell
MPP	Multipotent progenitor
mRNA	Messenger RNA
NF-KB	Nuclear factor-kappaB
NOD-SCID	Non-obese diabetic severe combined immunodeficiency
OS	Overall survival

PB	Peripheral blood
PCR	Polymerase chain reaction
PI	Propidium iodide
P-gp	P-glycoprotein
PI3K	Phosphatidylinositol 3-kinases
qPCR	Quantitative PCR
RNA	Ribonucleic acid
ROCK	Rho-associated, coiled-coil containing protein kinase
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SR-B1	Scavenge receptor-B1
SREBP	Sterol response element-binding protein
STAT5	Signal transduced and activator of transcription 5
ST-HSC	Short-term hematopoietic stem cell
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
ZGA	Zaragozic acid A

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Articles

K. van der Weide, P.M. Korthuis, F. Kuipers, E.G.E. de Vries, E. Vellenga. Hypoxia limits the cytotoxic effects of simvastatin in acute myeloid leukemia cells. *(manuscript in preparation)*

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'In vivo treatment of AML patients with high-dose simvastatin inhibits geranylgeranylation in AML cells.' *Blood* (ASH Annual Meeting Abstracts), 2010; 116: 3280.
(poster presentation)

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'Heterogeneity in simvastatin-induced cytotoxicity in AML is related to differential Ras-isoprenylation, rather than to blockade of cholesterol synthesis.' *(oral presentation)*

Annual meeting of the American Society of Hematology 2009, New Orleans, LA, USA
'Heterogeneity in simvastatin-induced cytotoxicity in AML is related to differential Ras-isoprenylation, rather than to blockade of cholesterol synthesis.'
Blood (ASH Annual Meeting Abstracts), 2009; 114: 1718. *(poster presentation)*

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(oral presentation)

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